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THE CYSTIC HYPERPLASIA-PYOMETRA COMPLEX IN THE BITCH

By

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INTRODUCTION

Pyometra is defined as an accumulation of pus in the uterus, but in the veterinary field the term is commonly applied to a post-oestrual syndrome of the adult bitch associated with a variety of clinical symptoms and pathological changes. A striking point which emerges from a study of the relevant literature is the paucity of information on the detailed pathology of the disease. The condition has been described under a variety of names such as hydrometra, pyometritis, catarrhal endometritis, chronic cystic endometritis, chronic sub-involution and many others. Since cystic glandular hyperplasia of the endometrium is found in a high percentage of cases, Low (1954) has suggested the use of the term "hyperplastic endometritis". This ignores the fact that the hyperplastic reaction need not necessarily be associated with inflammatory change. All these names have certain merits but since they describe only one feature or one stage of the disease process they lead to confusion.

The present study was undertaken to attempt a classification based on morphology of the various forms of the cystic hyperplasia-pyometra complex. In addition, it was hoped that a study of the histo-pathology of the disease might throw some light on the aetiological factors involved and so provide a basis for experimental reproduction of the condition.

MATERIALS AND METHODS

The material consisted of the ovaries, uterus and cervix from 100 cases of genital disease in the bitch. Seventy-nine were surgical specimens from cases of ovaro-hysterectomy performed in the hospital over a period of two and a half years. The remainder were obtained in the course of routine post-mortem examinations.

Tissues for microscopical examination were taken from both ovaries, from the upper, middle and lower thirds of each horn, from the corpus uteri and from the cervix. The blocks were fixed in sublimate-formol, dehydrated and cleared in an alcohol-amyl acetate-benzene series and double-embedded in celloidin and paraffin. Sections were stained as a routine with haemalum and eosin; other techniques used on selected tissues were picro-Mallory, Gordon and Sweet's reticulin stain, toluidine blue, Best's carmine, Southgate's mucicarmine, alcian blue, sudan IV, periodic acid-Schiff before and after hydrolysis with diastase, methylene blue in buffers of a range of pH values and pyronin-methyl green.

RESULTS

The series is divided into four major groups on a histological

basis. A comparison of the histochemical reactions of the endometrial mucin of the normal and pathological uterus is made. The ovarian changes are not described under each section but are given in a composite description.

Group I

Clinical observations. The mean age of the 23 cases, 15 of which were nulliparous, was $7 \cdot 1 \pm 2 \cdot 4$ years. The basic lesion was found in animals at all stages of the cycle. The only symptom was a slight mucoid vulval discharge which was present only in animals in metoestrus.

Morbid anatomy. The uteri varied in size with the degree of cystic glandular hyperplasia present, but only a few were grossly enlarged. In some specimens, the lumen contained a small amount of clear mucoid fluid. The endometrium was thickened and was lined by cysts of up to one and, occasionally, two cm. in diameter. In general, the cysts were scattered evenly throughout the length of the uterine horns but, in almost one-quarter of the number, they were localised to the upper ends of the horns. The endometrium of the corpus uteri was always less severely affected. Where there was a marked cystic hyperplasia the endometrium was thrown into folds and often formed small polypoid projections. Large single polyps were not observed. *Escherichia coli* was isolated from the uterine mucus in five cases which were in metoestrus; bacteria were not found in the uteri at other stages of the cycle.

Histology. Whereas, in any phase of the normal oestrus cycle, there is a striking uniformity in the size and configuration of the glands, in cystic glandular hyperplasia there is marked disparity. Some are large and cystic whilst others in the immediate vicinity are small and apparently normal (Fig. 1). The normal histological division of the endometrium into crypt, tubular and basal zones is lost. The larger cysts are generally round on cross-section but the smaller ones may be round, oval, star-shaped or branched. Although in some cases, it is possible to see two distinct layers of cysts, a basal layer of closely packed, small cysts and a superficial layer of larger cysts separated by an almost gland-free zone, in general the arrangement is less regular. Papillary projections into the lumina of the cysts are not uncommon. No direct correlation can be found between the size of the cysts and the stage of the cycle.

The superficial epithelium during anoestrus and pro-oestrus is similar to that seen in the normal animal, but as met-oestrus begins the increase in height and crowding of the cells is more marked. The gradual regression of the superficial epithelium, which is normally seen after the fourth week of met-oestrus is not apparent in cystic hyperplasia. The cells remain hypertrophied and pseudo-stratification is prominent with small foci of epithelial proliferation forming tuft-like projections into the uterine lumen (Fig. 2). The formation of crypts during oestrus is more prominent than normal

and in uteri examined after the first 20 to 30 days post-oestrus they are widely dilated so that the endometrial border has a scalloped outline. In many cases examined 30 to 50 days post-oestrus, the superficial and crypt epithelium has assumed a very florid, highly secretory appearance. The cells are tall and the nuclei are often nearer the luminal edge than the basement membrane. The cytoplasm is palely eosinophilic and finely vacuolated. In late met-oestrus, the cytoplasm of the superficial epithelium becomes almost completely vacuolated and fatty in appearance. The hyperchromatic nuclei in these cells are close to the luminal border. This process is seen in normal animals in late met-oestrus, but it does not extend to the crypt epithelium as it does in cystic hyperplasia.

The morphology of the cyst epithelium varies throughout any one section and rarely resembles that seen in normal uterine glands at a comparable stage in the oestrus cycle. Low cuboidal epithelium is seen in many cysts, particularly the smaller ones, even during the early met-oestrus phase when secretion is normally at its height. In many such cysts secretion may not be observed on paraffin sections, whilst in others the lumen is filled with a pale eosinophilic fluid. Neighbouring glands may exhibit a tall, secretory epithelium such as is seen in normal met-oestral glands. In some cysts an unusual degree of proliferation of the lining epithelium may be seen with pseudo-stratification similar to the florid type of superficial epithelium. Such cysts are usually superficially placed and may form polypoid projections into the uterine lumen. It is not uncommon to find cysts in which the epithelium nearest the uterine lumen is tall and highly secretory, but gradually decreases in height to become cuboidal in the base of the cyst.

Stromal proliferation is rarely pronounced; in most cases, cystic glandular hyperplasia predominates and may completely fill the endometrium, leaving only a thin supporting connective tissue stroma. An impression of increased stromal cellularity may be obtained where cystic dilatation of glands causes compression of the stroma. Mitotic figures are rare among the stromal cells. The reticulin fibres around the cysts are intact, but are compressed into a denser network. The collagen fibres of the endometrial stroma are normal in number and structure, but are arranged in a more distinctly peri-glandular fashion than normal. There is no evidence of inflammation in any part of the endometrium.

The myometrium is hypertrophied in only a small number of the specimens examined. The vessels of the stratum vasculare are dilated and tortuous in those cases which are in metoestrus. In seven cases there is adenomyotic extension of endometrial glands into the myometrium. The aberrant endometrial tissue extends through the inner layer of muscle as far as the stratum vasculare, but in no instance can it be seen in the outer myometrium (Fig. 3). The adenomyotic glands show the same changes as those in the endometrium and in four cases there is considerable cystic dilatation.

Group II

Clinical observations. The average age of the 17 cases in this group was 7·2 ±1·9 years. All the animals were in the period 40 to 70 days after oestrus. Only four bitches had borne litters. Symptoms were generally mild: in most only a mucoid vulval discharge was noted. Some cases had a slight neutrophilia.

Morbid anatomy. The uteri were hypertrophied, but rarely exceeded two cm. in diameter. The uterine walls were thickened and the endometrial surface was covered with irregular cysts and small polypi. The endometrium was a dull greyish colour and the cysts were less translucent than those in the previous group. The uterine lumen contained a small amount of mucoid fluid, usually clear, but occasionally slightly brown or pink. The cervical canal constantly allowed passage of a probe of 0·3 cm. diameter or more and organic lesions of the cervix were not observed in any case.

No bacterial growth was obtained on culture of fluid from three of these uteri and the remainder yielded *E. coli.*

Histology. The basic morphological picture of the endometrium is that of cystic glandular hyperplasia with infiltration of the surrounding stroma by plasma cells.

The superficial epithelium is of a florid highly secretory type with a marked tendency to undergo stratification, forming small fan-shaped tufts of cells at various points or broad sheets of up to ten cells thick extending into the uterine lumen. Many of the superficial glandular cysts are lined by a similar epithelium which gives a scalloped outline to their lumina. The cysts of the basal region of the endometrium are generally smaller and of a more uniform size. They are lined by a low columnar or cuboidal epithelium, the cytoplasm of which rarely contains secretory vacuoles. All the cysts contain a variable amount of secretion which is commonly cell-free. The cellular infiltration of the endometrium is almost entirely composed of plasma cells with a few lymphocytes, macrophages and an occasional mast cell. Polymorphonuclear leucocytes are rare. The plasma cells are most frequently concentrated in the superficial stroma though, in some cases, there is diffuse infiltration throughout all the layers of the endometrium (Fig. 4). Where the plasma cells are confined to the surface areas of the endometrium the stroma of the basal zone is oedematous. The basal zone is always highly vascular and moderately large vessels can be seen extending towards the surface of the endometrium. The superficial capillaries are dilated, but there is no evidence of haemorrhage though occasional haemosiderin-containing macrophages are visible. There is no necrosis or atrophy of any endometrial structure. In 14 cases, there is no fibroblast proliferation nor is there any change in the number, type or arrangement of the collagen fibres. In two cases there is some increase in the number of fibroblasts in the tubular and crypt zones. The reticulin network of the crypt zone in

these two specimens is less extensive and collagen fibres are more numerous and coarser. In the final case, circular orientation of thick collagen fibres in the superficial endometrium is prominent. The reticulin network around the crypts and superficial cysts has been largely replaced by collagen fibres, but the basement membrane is still visible. The basal stroma is oedematous and shows no evidence of fibrosis.

The inner, circular layer of the myometrium is hypertrophied and there is often some oedema of the stratum vasculare, the vessels of which are dilated. The perivascular connective tissue of the inner myometrium contains a variable number of plasma cells which are particularly numerous in those cases where endometrial infiltration is diffuse. In three cases adenomyotic foci are present in the stratum vasculare.

The cervical mucosa is oedematous in the seven cases examined, but cellular infiltration is slight. A few plasma cells, lymphocytes and neutrophils are visible in the epithelium and in the immediate subjacent connective tissue. There is no evidence of fibrosis.

Group III

Clinical observations. The mean age of the 49 cases in this group was $8 \pm 2 \cdot 2$ years. Forty were nulliparous and only three had borne more than one litter. The interval between the last pregnancy and the onset of symptoms was at least four years in the parous bitches. The majority of the animals became ill during the first 40 days after oestrus. A mucopurulent, often blood-tinged vulval discharge was observed in 75 per cent of the animals. All were dull, listless and inappetant. Thirst and vomiting were frequent symptoms. Haematological examination revealed a leucocytosis with white cell counts ranging from 19,000 to 145,000 per cu. mm. The erythrocyte sedimentation rate was always accelerated, but anaemia was not observed.

Morbid anatomy. Uteri weighing up to four kg. and with horns measuring up to 50 cm. in length and up to seven or eight cm. in diameter were not uncommon in 15 to 20 kg. bitches. Where the uterine horns were grossly enlarged they were forced into coils by the restraining action of the broad ligament. The horns were either of uniform diameter throughout their length or, more often, exhibited a number of annular constrictions producing a series of irregular ampullae. The constrictions were usually slight, but in some instances were so marked that a series of non-communicating loculi was produced. The corpus uteri rarely showed the same degree of distension as the uterine horns and, in some cases, appeared of little more than normal diameter. In three specimens only one horn was distended, the contralateral horn being slightly hypertrophied or of normal proportions. The wall was hypertrophied in most of the uteri examined, but in some grossly distended specimens decrease in thickness of the wall was evident. The cervical canal was

completely closed in eight of the specimens and, in many others, the os was narrow and fluid could be extruded only by the application of considerable pressure.

The uterine fluid varied from a few ml. up to two litres and was yellow or green in some cases but more often was tinted red or brown with blood. The viscosity of the fluid varied considerably; it was generally lowest when haemorrhage was present and highest where the fluid was yellow or green. Severe haemorrhage was not observed in any of the specimens examined. The endometrium had a roughened appearance, with mingling of pus-filled cysts, focal haemorrhages and areas of ulceration. Thick muco-pus was often adherent to the areas of ulceration, which were most prominent in grossly distended uteri. Areas of localised peritonitis were noted in eight cases and in one case an acute peritonitis had followed rupture of the uterine wall.

Only five cases failed to yield bacteria on cultural examination of the uterine fluid. *E. coli* was the commonest organism and was isolated from 40 cases. Six cases yielded a coagulase positive staphylococcus and five a β-haemolytic streptococcus.

Histology. The characteristic feature of this group is the presence of an acute inflammatory reaction in an endometrium which shows a variety of stromal and glandular changes. In many specimens the cystic glandular hyperplasia is divided into two morphologically distinct layers, superficial and basal. The superficial cysts are very irregular in outline and are lined by an epithelium which, in general, is similar to that of the crypts and endometrial surface. The epithelial cells are tall and of a secretory type. They may be in a single layer, but frequently the arrangement is more complex. Pseudo-stratification may be focal, producing numerous small epithelial papillae, or it may be diffuse forming syncytium-like sheets. The basal cysts are always more regular in outline. They are lined by a simple cuboidal epithelium in most instances though occasional groups of cysts lined by tall, secretory cells are seen.

In mild cases, cellular infiltration is confined to the superficial half of the endometrium. Numerous neutrophils are present in the stroma beneath the superficial epithelium and around the crypts and adjacent cysts (Fig. 5). Infiltration of the epithelium is particularly prominent in areas of stratification and the uterine lumen contains large numbers of neutrophils mixed with secretion. Inflammatory exudate into the crypts and cysts is irregular. Some contain only mucin, but others are filled with neutrophils and cell debris. Necrosis of some of the small endometrial polypi is seen at this stage. The basal half of the endometrium shows no evidence of neutrophil infiltration. The stroma is very oedematous and congestion of the basal vessels is evident.

In more severely affected cases, the neutrophil infiltration extends into the basal half of the endometrium. Many cysts become completely filled with inflammatory exudate and degenerative changes

are visible in the lining epithelium. Abscesses are frequently formed around cysts in which the lining epithelium has undergone necrosis. These basal abscesses may extend to occupy the entire width of the endometrium or they may ulcerate into the uterine lumen. In many grossly distended uteri, ulceration is widespread and complete sloughing of the entire width of the endometrium may occur at some points. Ulceration may also be seen in association with thrombosis of some of the basal arteries. In the base of small isolated ulcers there is sometimes evidence of early granulation tissue formation. Plasma cells are present in every specimen examined in this group. Their number and distribution is similar to the lesions described in Group II. Eosinophils are present in many cases but are never numerous.

Evidence of fibrosis is present in only five cases, three of which are known to have had previous attacks of post-oestral endometritis. The fibrosis in all cases appears as a broad band of connective tissue arranged in a circular manner. In two specimens the fibrous tissue, which contains a few glandular lumina, lies between the superficial and basal cysts. The superficial zone is diffusely infiltrated by neutrophils, but the area beneath the fibrous tissue shows no inflammatory reaction. In the other cases there is only a narrow lamina propria of relatively fine connective tissue separating the zone of fibrosis from the superficial epithelium (Fig. 6). There is intense neutrophil infiltration of the lamina propria and of the subjacent fibrous tissue with ulceration at many points. The basal zone shows cystic glandular hyperplasia, hyperaemia and oedema.

In 50 per cent of the uteri examined there is extension of the acute inflammatory process to the myometrium. Where ulceration is severe there is necrosis of muscle fibres and diffuse neutrophil infiltration of the myometrium in the base of the ulcer. These ulcers rarely extend far into the inner, circular layer of the myometrium; in only one case has complete perforation occurred. The commonest form of myometrial involvement is extension of the inflammatory reaction along the perivascular connective tissue. The vessels penetrating the inner myometrium are commonly surrounded by cuffs of neutrophils and plasma cells. The stratum vasculare is always oedematous, and the vessels in it are dilated and show increased tortuosity like that seen in normal oestrus. Abscesses of the stratum vasculare are not uncommon and infiltration of the neighbouring vessels is sometimes seen. Thrombosis of these vessels is present in only one case. It is of interest that one specimen shows typical lesions of polyarteritis nodosa in the arteries of the uterus, ovaries and broad ligaments in association with a mild acute endometritis. Muscle biopsies taken six weeks later reveal that the condition was not generalised. In most cases the outer muscle layer is hypertrophied. Where the uterine distension is gross, degenerative changes in the muscle fibres are evident. There is atrophy of muscle fibres with loss of staining affinity.

The lamina propria of the cervix is commonly oedematous and shows a cellular infiltration similar to that of the endometrium, but rarely of the same severity. Evidence of fibrosis cannot be found in any of the specimens examined.

Group IV

Clinical observations. The mean age of the 11 animals in this group was $11 \cdot 8 \pm 1 \cdot 7$ years. Only two bitches had been pregnant and in these, the interval between parturition and examination was more than six years. A history of previous attacks of post-oestral endometritis was obtained in nine. The disease was divided into two distinct clinical types, mild and severe. In four cases a muco-purulent discharge was evident, but systemic upset was slight. Total leucocyte counts ranged from 16,000 to 21,000 cells per cu. mm. In the other animals, there was marked abdominal distension, anorexia, vomition and prostration. In these, the total leucocyte counts were from 31,000 to 68,000 cells per cu.mm.

Morbid anatomy. The uteri were divided into two pathological sub-groups corresponding to the clinical sub-division. In the seriously ill animals the cervix was tightly constricted, but was not obviously enlarged. The uterine horns were grossly distended and the walls were so thin that it was impossible to differentiate endometrium and myometrium. The uteri contained up to 2·5 litres of thin watery brown fluid. The endometrial surface was grey with occasional flecks of haemorrhage and it had a finely granular appearance. Low irregular ridges were apparent at intervals on the endometrial surface but only occasional cysts were observed. In the mild cases the cervix was patent and the uterine horns were never more than three cm. in diameter. The uterine walls were slightly hypertrophied in two cases, but increase in thickness was marked in the other two. The endometrial surface appeared almost normal except for the presence of occasional polypi.

On cultural examination, two of the "closed" cases did not yield organisms. *E. coli* was isolated from eight uteri, *Staph. aureus* from two and a β-haemolytic streptococcus from two.

Histology. The microscopic picture in the two sub-groups is as distinct as the clinical and gross pathological features.

In the distended uteri, only a thin lamina propria separates the superficial epithelium from the inner layer of the myometrium. The superficial epithelium in four cases is of low cuboidal or pavement type, becoming slightly taller in the thicker parts of the endometrium. In the other three cases, the superficial epithelium is of a stratified squamous type of up to ten cells thick at all levels of the uterus. At some points there are a few glands, but generally they are rare. Squamoid change is present in the glands as well as in the superficial epithelium in two cases (Fig. 7). The thin lamina propria in all these cases is composed of circularly arranged collagen fibres which are closely packed. There is a diffuse infiltration of the endometrium by

plasma cells and lymphocytes in all cases. The bases of the occasional ulcers contain highly vascular granulation tissue, but in general, hyperaemia of the endometrium is not marked. In one case, considerable numbers of neutrophils have infiltrated the stratified squamous epithelium.

Both layers of the myometrium are atrophic in these grossly distended uteri. The muscle fibres are thin and have lost their staining affinity. The nuclei are slender, elongated and hyperchromatic. There is diffuse plasma cell and lymphocyte infiltration of the myometrium in most of the specimens. The cervical epithelium is normal in structure. The lamina propria contains a small number of plasma cells and lymphocytes. There is an increase in the number of collagen fibres in the lamina propria. They are coarse and are arranged in a distinctly circular manner. There is a mild degree of fibrosis of the cervical muscle in two cases.

In the mild cases with uteri which were not distended with pus the endometrium is atrophied (Fig. 8). The superficial epithelium is composed of low cuboidal cells or of the fatty degenerate type seen in normal late metoestrus. Crypts are not numerous and the endometrium is rarely more than two glandular lumina in width. Occasional polypoid cysts are seen, but most of the small papillomata which protrude into the uterine lumen do not contain glandular elements. There is a mild diffuse infiltration of the endometrium by plasma cells and lymphocytes. There is some increase in the number of collagen fibres, particularly around the glands and in the superficial half of the endometrium.

There is an increase in the amount of connective tissue around the muscle bundles in both layers of the myometrium. The connective tissue is less cellular and is composed of thick collagen fibres. The muscle fibres are hypertrophied in some cases, whilst in others the fibres are atrophic and the myometrium is fibrosed. Perivascular cuffing by lymphocytes and plasma cells is prominent in the vessels traversing the inner layer of muscle. The cervical mucosa is infiltrated by round cells, but there is no evidence of fibrosis.

Histochemical Examination of Endometrial Mucin

The staining reactions of the glandular mucin in the normal and in the pathological endometrium are shown in Table 1. The results given for Group IV are true only for a minority since secretion is not present in the glands or superficial epithelium of most cases. In the normal endometrium, the mucin is present as fine intracytoplasmic granules in the crypt and glandular epithelium during pro-oestrus, oestrus and early metoestrus, but after 15 to 20 days of metoestrus it is almost entirely in the lumina of the glands. In many cases of the cystic hyperplasia-pyometra complex, intracytoplasmic mucin is still visible up to the 50th day post-oestrus. In others, it is entirely in the uterine and glandular lumina. There is no obvious difference in the composition of the mucin in the normal

endometrium and that in various pathological uteri examined. The findings indicate that the endometrial secretion contains acid muco-polysaccharide.

Sudanophilic material is present only in the cytoplasm of the large degenerate superficial cells seen in the latter half of metoestrus and in a few cases of chronic endometritis.

TABLE 1

HISTOCHEMICAL REACTIONS OF ENDOMETRIAL MUCIN IN THE BITCH

Material	*Normal*	*Group I*	*Group II*	*Group III*	*Group IV*
P.A.S.	+	+	+	+	+
Diastase+P.A.S.	+	+	+	+	+
Metachromasia with Toluidine blue	+	+	+	+	+
Methylene blue Extinction.. ..	pH2	pH2	pH2	pH2	pH2
Mucicarmine	+	+	+	+	+
Alcian blue	+	+	+	+	+
Picro-Mallory	—	—	—	—	—
Sudan black B	—	—	—	—	—

Ovary

No group specific changes were observed and a composite description is given.

Morbid anatomy. Eighty-eight cases were in metoestrus and 80 of these had recognisable corpora lutea in at least one ovary. In many cases in the latter half of metoestrus the corpora lutea appeared larger than in normal animals at a comparable stage of the cycle. In three cases, both ovaries were transformed into multiloculated cysts up to seven cm. long, which were thin-walled and contained a clear, watery fluid. In two cases multicystic change was observed in only one ovary; the other contained corpora lutea. Single cysts of one to three cm. in diameter were present in a further 11 specimens. In three cases, one ovary contained a solid, lobulated mass two to three cm. in diameter. These masses were homogeneous, white and cellular in appearance. Corpora lutea were present in the contralateral ovary in two cases and in the third, the ovary was small and smooth.

In those cases of uncomplicated cystic hyperplasia which were not in metoestrus, the ovaries were generally smooth and apparently inactive. One ovary had undergone cystic change in two cases. In one case, one ovary contained a tumour-like mass four cm. in diameter and the other ovary contained several polypoid cysts one to two cm. in diameter.

Histology. Active or regressing corpora lutea are present in one or both ovaries of 96 cases. Personal observations and those of Mulligan (1942) show that in the normal animal the granulosa lutein cells reach their greatest size about ten days after ovulation. The cells are large and oval, with pale eosinophilic cytoplasm stippled with tiny vacuoles. The nucleus occupies about one fifth of the cell and is large and vesicular with a prominent nucleolus. The central cavity rapidly fills with lutein cells, fine connective tissues and slender vessels. In the non-pregnant bitch, the first signs of regression appear in the corpus luteum between the 25th and 30th days of metoestrus. There is a gradual increase in the amount of connective tissue and shrinkage in the total size of the corpus luteum. Many of the granulosa lutein cells become more angular and the cytoplasmic vacuoles become larger and more numerous. The amount of cytoplasm relative to nuclear size decreases. Some nuclei become more condensed and hyperchromatic. By the end of metoestrus, the corpus luteum is broken up by connective tissue trabeculae and is composed of small cells with hyperchromatic nuclei and completely vacuolated cytoplasm. The corpora lutea of pregnancy exist throughout gestation as well-developed, highly vascular structures and degenerate rapidly after parturition. By the 90th day after ovulation, it is impossible to differentiate the corpora lutea of pregnancy from those of metoestrus.

In cases of the cystic hyperplasia-pyometra complex the corpora lutea appear morphologically consistent with the estimated stage of the cycle for the first four weeks after oestrus. In clinical cases arising later in metoestrus, it is common to find corpora lutea which have not undergone the expected degree of regression. There is only moderate connective tissue infiltration and the luteal tissue is highly vascular. Though some of the lutein cells do show retrogressive changes, the majority appear of maximum size even as late as 50 days post-oestrus. These corpora lutea resemble those of a comparable stage of pregnancy more closely than those of normal metoestrus. This apparent persistence of luteal function is particularly prominent in Group III and in several cases of Group IV. The corpora lutea of cases in Group II show evidence of regression though this is frequently not of the degree expected for the stage of cycle. There appears to be a correlation between the duration of persistence of the corpora lutea and the complexity and secretory activity of the endometrial epithelium. The corpora lutea in Group I are morphologically consistent with the estimated stage of the cycle.

The cysts which are present in 19 cases are thin-walled structures lined by one or several layers of epithelium surrounded by remnants of the theca interna or by compressed ovarian stroma. In some single cysts, it is possible to recognise a lining of several layers of granulosa cells, but in most cases, there is only a single layer of low columnar or cuboidal cells. In the larger cysts and in the polycystic variety, the lining epithelium is flattened and the nuclei are

dense and hyperchromatic. In some the epithelium has disappeared and the cysts are lined by fibrous connective tissue. Even in some ovaries of the polycystic variety in which only small islands and trabeculae of ovarian tissue remain all stages of follicle growth and regression can be seen. No normal follicular or luteal structures can be found in two polycystic cases associated with uncomplicated cystic glandular hyperplasia. One unilateral polycystic mass proves to be a pseudo-mucinous cystadenoma lined by characteristic tall clear cells with dark-staining nuclei. The contralateral ovary contains functional corpora lutea.

Granulosa cell tumours are present in eight cases though only four were considered abnormal at post-mortem examination. The tumour is bilateral in one case. The microscopic pattern is extremely variable even in individual tumours though the cells usually resemble granulosa cells. In the majority, the microfolliculoid pattern predominates with areas of diffuse, solid growth in some cases. Call-Exner rosettes are common in some tumours. In two tumours connective tissue ingrowth has produced a cylindromatous arrangement. Inflammatory changes in the ovaries are absent except in one case of polyarteritis nodosa. The uteroovarian vessels in the hilar area of the ovaries are very tortuous and dilated in cases of endometritis.

DISCUSSION

From the results of this survey it is clear that the cystic hyperplasia-pyometra complex is primarily a disease of the older, nulliparous bitch which becomes clinically manifest during the luteal phase of the oestrus cycle. It differs in many respects from cystic hyperplasia and pyometra in the human. Fluhmann (1931) interpretes endometrial hyperplasia in women as a manifestation of overstimulation of the uterus by oestrogens in the absence of progesterone activity. Novak (1952) states that secretory activity is rare in genuine hyperplasia of the human endometrium, whereas in the canine cases reported here mucin production appears to be directly proportional to the degree of hyperplastic change. Babes (1924) observed corpora lutea in the ovaries of 3 out of 24 human patients with endometrial hyperplasia. In the present study of the canine disease corpora lutea were present in 96 per cent of the ovaries examined. This association of corpora lutea and endometritis in the bitch was first noted by De Vita (1939), but among later workers only Teunissen (1952) has attached any significance to it. Witherspoon (1935) recorded the presence of ovarian cysts in 45 per cent of his cases of cystic hyperplasia in women and postulated a relationship between follicular cysts and endometrial hyperplasia. Though Hetzel (1935) and Bloom (1954) have suggested that a similar relationship exists in the dog, the results of the present survey do not support this contention since ovarian cysts were found in only

19 per cent of the cases examined. In only 2 cases did ovarian cysts occur in the absence of corpora lutea.

Bland (1929), discussing pyometra in women, listed as possible causes a variety of conditions producing partial or complete obstruction of the cervical canal. He stressed the frequent association of the disease with fibrosis and tumours of the cervix. Complete closure of the cervix was observed in only 15 of the 100 cases examined in the present series, though in many others the patency of the canal was only sufficient to allow discharge of a fraction of the uterine fluid. In no case was occlusion of the cervix due to blockage by tumour. Fibrosis of the cervix was recorded only in a few chronic cases. Since spontaneous opening or closing of the cervix may occur during the course of the disease in the bitch, it would appear that the cervical closure is commonly functional rather than anatomical. Occlusion of the cervical canal is normal during metoestrus in the bitch. Spontaneous pyometra without organic lesions of the cervix has been recorded in women (Graham and Failla, 1940).

Wolfe, Campbell and Burch (1932) showed that cystic hyperplasia of the endometrium could be produced in guinea-pigs and rats by the administration of oestrogens. Similar results were obtained in the rabbit and mouse by Lacassagne (1935). Hetzel (1935) produced a mild hyperplasia of the endometrium in bitches treated with oestrone and from this inferred that the natural condition is a manifestation of hyperoestrinism. Bloom (1954) subscribed to this view, but recognised that corpora lutea are frequently associated with the disease. He suggested the following explanations of this inconsistency:— (1) the hyperplastic endometrium may be refractory to progesterone; (2) the corpora lutea may be non-functional; (3) the production of oestrogen may be so great that progesterone exerts no balancing effect.

All three possibilities are refuted by the observations made in the present study. In all cases of endometritis histological examination of the ovaries reveals corpora lutea which appear active. The secretory changes present in these uteri are identical with those produced by progesterone. The third explanation seems unlikely because there is no clinical or pathological evidence of hyperoestrinism. From this survey it is concluded that the presence of corpora lutea is of significance in the pathogenesis of the canine cystic hyperplasia-complex. Experimental results, to be published at a later date, support this conclusion.

CONCLUSIONS

A survey of 100 cases of the cystic hyperplasia-pyometra complex in the bitch is given. The cases are divided into four subdivisions on a histological basis.

In Type I (23 cases) there was cystic hyperplasia in which cellular infiltration of the endometrial stroma was absent. This lesion was observed in animals in different stages of the cycle.

In Type II there was a diffuse plasma cell infiltration superimposed on cystic glandular hyperplasia of the endometrium in 17 cases. This lesion was found only in animals between 40 and 70 days post-oestrus.

In Type III there was a polymorphonuclear infiltration of varying intensity superimposed on a cystic endometrium in 49 cases. The majority of these cases were observed in the first 40 days after oestrus.

In Type IV (11 cases) there was a chronic endometritis, often with squamous metaplasia of the epithelium. These were observed in the period 55 to 90 days after oestrus.

Corpora lutea were observed in the ovaries of 96 cases and it is concluded that this is of pathogenetic significance.

ACKNOWLEDGMENT

The author wishes to record his indebtedness to Dr. W. F. H. Jarrett for his advice and criticism.

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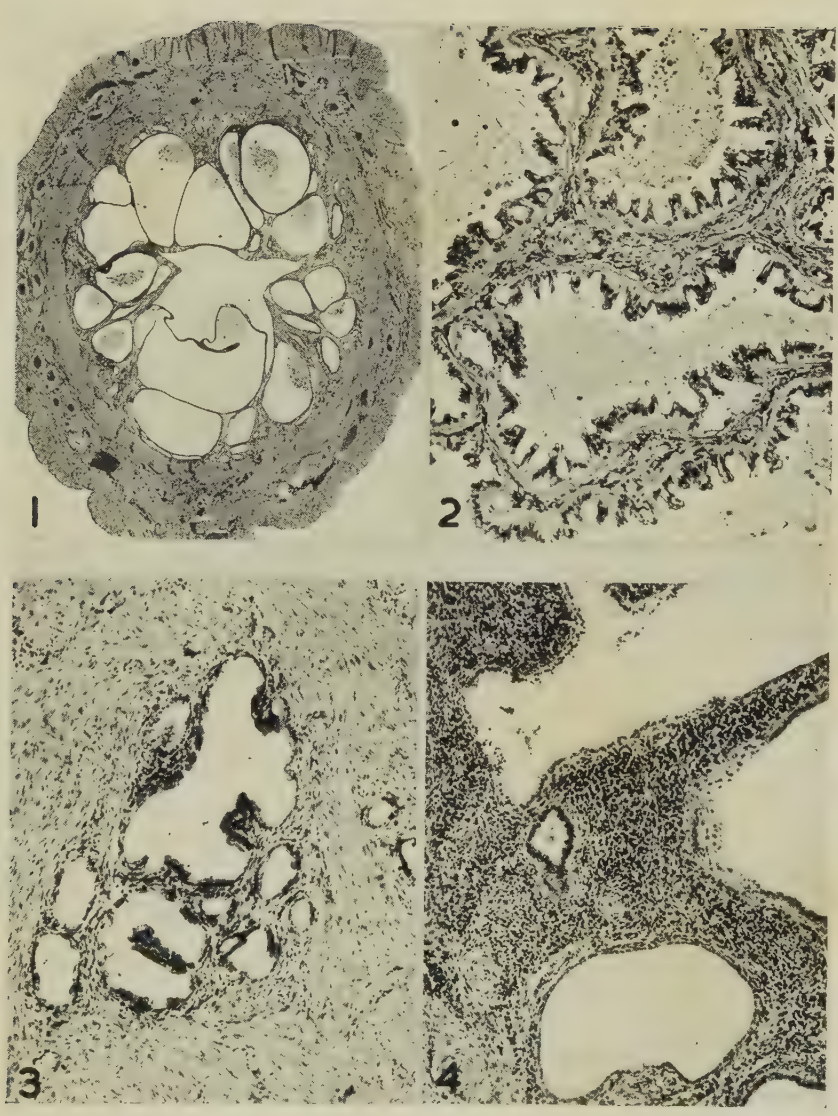

Fig. 1. Cystic glandular hyperplasia of the endometrium. H. & E. × 6.

Fig. 2. Cysts lined by tall columnar epithelium; marked subnuclear vacuolation of the cytoplasm. H. & E. × 90.

Fig. 3. Focus of endometrial glands in the inner myometrium. H. & E. × 90.

Fig. 4. Cystic glandular hyperplasia of the endometrium; diffuse plasma cell infiltration of the stroma. H. & E. × 90.

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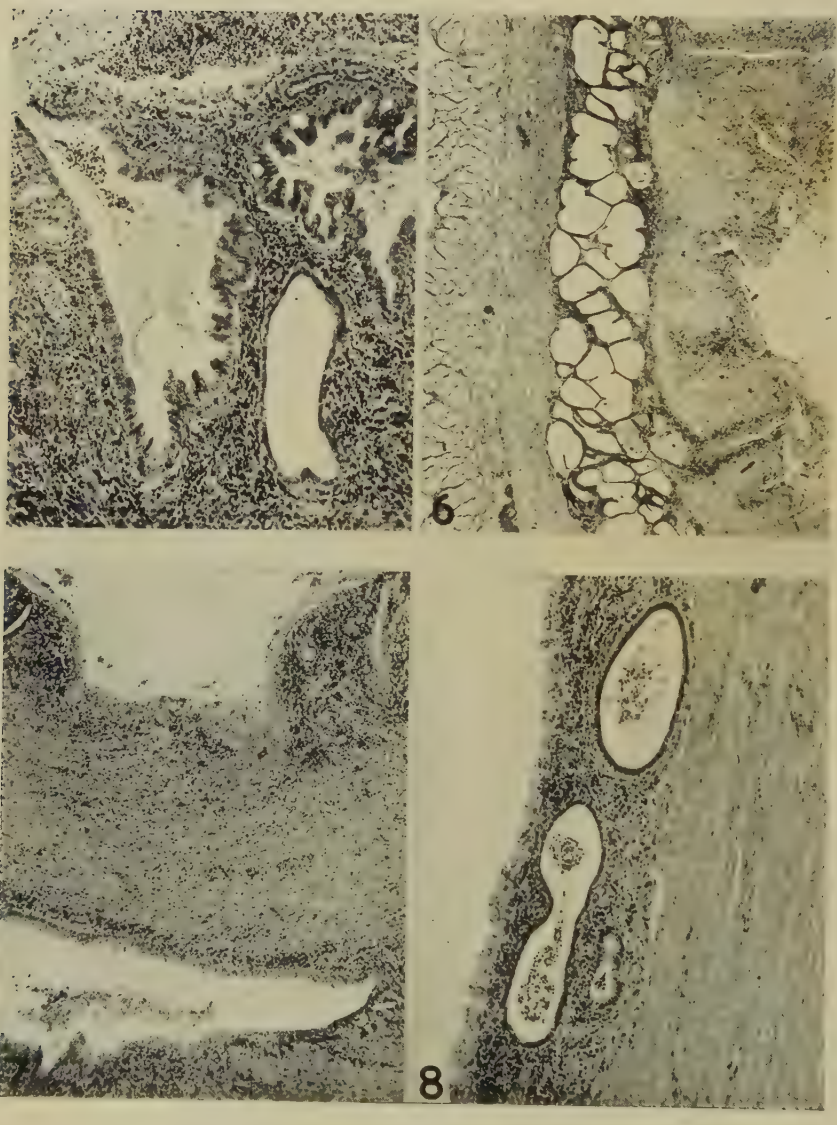

Fig. 5. Cystic glandular hyperplasia with a superimposed acute endometritis. H. & E. × 90.

Fig. 6. Acute endometritis with fibrosis of the superficial half of the endometrium and cystic dilatation of the basal glands. H. & E. × 8.

Fig. 7. Chronic endometritis with squamous metaplasia of the superficial and glandular epithelium. H. & E. × 27.

Fig. 8. Chronic endometritis; atrophy of the endometrium and myometrium; round cell infiltration of the endometrium. H. & E. × 90.

THE USE OF CORTISONE-TREATED MICE FOR THE EVALUATION OF *BRUCELLA ABORTUS* (S.19) VACCINE

By

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INTRODUCTION

De Ropp (1945) studied the virulence of *Brucella abortus* (Strain 544) in the mouse. He found that an infecting dose of 300,000 viable organisms in no instance caused death, but only a chronic type of infection difficult to characterise. Coid (1955) found that 2000 $\times$ 10^6 organisms were necessary to produce death with the same strain. The mouse has thus not so far been regarded as suitable for standardising S.19 vaccine and the guinea pig has had to be used. This, however, involves time-consuming tests over sixteen weeks. Further, the final assessment of results may be difficult, since it can be based on three distinct criteria, namely, recovery of *Br. abortus* from the spleens, ratio of spleen-weight to body-weight and serum agglutination titre.

Recently attention has been directed towards the practicability of making laboratory animals more susceptible to infective agents by pre-treatment with cortisone, a technique already proved of value in studies with poliomyelitis virus (Syverton, Brunner, Tobin and Cohen, 1956). It was thought that, if the sensitivity of mice to *Br. abortus* could be increased by this means, it might then be possible to use them for the assay of S.19 vaccine and thereby avoid the disadvantages of the guinea pig test.

MATERIALS AND METHODS

Animals. The laboratory strain of inbred albino mice (Strong A2/G) was used, male animals of 20g. weight being selected. Guinea pigs were obtained from a registered breeder and were of either sex, weighing approximately 200 g.

Cortisone. A suspension of cortisone acetate* in saline was prepared to contain 15 mg. per ml. Mice were given subcutaneously 9 mg of cortisone divided into three equal doses at intervals of 48 hours. The last dose was given 5 hours before challenge with *Br. abortus*.

Challenge strain. The challenge strain was S.544 known to be virulent for cattle (McEwen, Priestley and Paterson, 1939) and guinea pigs (De Ropp, 1945) and recommended in the W.H.O. Report No. 19 (1943) for the standardisation of S.19 vaccine. A suspension of the strain was prepared in Ringer's solution from a 48 hour Albimi agar slope culture, grown at 37°C in an atmosphere of 5 per cent carbon dioxide. The opacity of the suspension was adjusted to Brown's tube 10; a viable count was made by the method of Miles and Misra (1938).

* "Cortelan", Glaxo Laboratories Ltd., Greenford, Middlesex

Vaccine. Freeze-dried S.19 vaccine was supplied by the Ministry of Agriculture, Fisheries and Food Laboratories, Weybridge. The vaccine was suspended in 5 ml of standard diluent. Doses of 4 to 8 $\times$ 10^6 organisms contained in 0·2 ml were used to vaccinate mice subcutaneously and 400 to 800 $\times$ 10^6 organisms contained in 1·0 ml to vaccinate guinea pigs intramuscularly.

RESULTS

Influence of Cortisone on the Susceptibility of Mice to Br. abortus *(S.544)*

A group of 70 mice received cortisone treatment, a further 50 remaining as controls. Five hours after the last dose of cortisone, 50 of the treated mice and the 50 untreated mice were divided into five pairs of groups of 10 mice and each group received intraperitoneally tenfold dilutions of S.544 ranging from 700 $\times$ 10^6 to 0·07 $\times$ 10^6 viable organisms in 0·5 ml. The remaining 20 mice served as controls. The mice were observed for 14 days, during which all deaths were recorded, and an $L.D._{50}$ was calculated by the Reed-Muench formula. From Table 1 it will be seen that the $L.D._{50}$ of S.544 for mice previously treated with cortisone was at the dilution of $10^{-4.6}$, which represented approximately 0·3 $\times$ 10^6 viable organisms. In the mice receiving no cortisone only one death occurred in the group receiving the highest number of organisms. No deaths occurred in the non-challenged cortisone-treated mice. These results show that pre-treatment with cortisone increased the susceptibility of the mice to S.544 at least one-thousand fold.

TABLE 1

INFLUENCE OF CORTISONE IN INCREASING THE SUSCEPTIBILITY OF MICE TO *BR. ABORTUS* (S.544)

Cortisone dose	*Challenge S.544*	*Survivors Total challenged after 14 days*
3 $\times$ 3 mg (given at 48 hour intervals)	700 $\times$ 10^6	0/10
	70 $\times$ 10^6	0/10
	7 $\times$ 10^6	0/10
	0·7 $\times$ 10^6	3/10
	0·07 $\times$ 10^6	6/10
as above	not challenged	20/20
no treatment	700 $\times$ 10^6	9/10
	70 $\times$ 10^6	10/10
	7 $\times$ 10^6	10/10
	0·7 $\times$ 10^6	10/10
	0·07 $\times$ 10^6	10/10

Influence of Cortisone on Mice Subsequently given Br. abortus *S.19 vaccine*

De Ropp (1945) concluded that the mouse was even less sensitive to S.19 than to S.544. It was necessary, however, to see if the

administration of cortisone would increase the susceptibility of mice to S.19; if this were so, it would preclude the use of the mouse as a subsequent test animal for evaluating S.19 vaccine. In this experiment 56 mice were divided into two equal groups, one group receiving cortisone treatment, and both were then divided into four sub-groups each of 7 mice. Each sub-group was subsequently challenged subcutaneously with ten fold dilutions ranging from 4000×10^6 to 4×10^6 organisms. The mice were observed for fourteen days and all deaths were recorded. From Table 2 it will be seen that cortisone did not markedly increase the susceptibility of the mice to S.19.

TABLE 2

INFLUENCE OF CORTISONE ON MICE SUBSEQUENTLY GIVEN *BR. ABORTUS* (S.19)

Cortisone treatment	*Challenge S.19*	*Survivors Total challenged after 14 days*
3 × 3 mg given at 48 hour intervals	4000×10^6	4/7
	400×10^6	7/7
	40×10^6	7/7
	4×10^6	7/7
no treatment	4000×10^6	7/7
	400×10^6	7/7
	40×10^6	7/7
	4×10^6	7/7

TABLE 3

EVALUATION OF S.19 VACCINE ON THE MOUSE

Vaccine (S.19)	*Cortisone dose*	*Challenge (S.544)*	*Survivors Total challenged after 14 days*
0·2 mls ($4–8 \times 10^6$)	3 × 3 mg at 48 hour intervals	100 $L.D._{50}$	20/20
as above	as above	not challenged	20/20
not vaccinated	as above	100 $L.D._{50}$	0/20

Evaluation of S.19 Vaccine on Mouse and Guinea pig

Forty mice and twelve guinea pigs were vaccinated. Three weeks after vaccination the mice, along with twenty non-vaccinated controls, received cortisone. Twenty of the vaccinated mice and the twenty non-vaccinated controls were subsequently challenged intraperitoneally with 100 $L.D._{50}$ of S.544 contained in 0·5 ml.

TABLE 4

EVALUATION OF S.19 VACCINE ON THE GUINEA PIG

Guinea pig No.	*Treatment*	*Criteria of infection*					
		Ratio spleen wt. body wt.	*Mean ratio spleen wt. body wt.*	*Spleen count*	*No. + spleens total*	*Agglutination titre*	*Mean agglutination titre*
A1	vaccinated	0·155		0		20	
2	and	0·196		0		0	
3	challenged	0·3		0		40	
4		0·196		0		40	
5		0·178		0		40	
6		0·214	0·2	0	1/12	40	20–40
7		0·245		0		20	
8		0·197		0		0	
9		0·177		0		0	
10		0·235		200		40	
11		0·176		0		20	
12		0·185		0		20	
B1	non-vaccinated	0·41		100		160	
2	and	0·46		200		320	
3	challenged	0·19	0·33	20	6/6	40	80–160
4		0·34		100		40	
5		0·39		30		80	
6		0·195		5		160	
C1	non-vaccinated	0·176		0		0	
2	and	0·192		0		0	
3	not challenged	0·245	0·21	0	0/6	0	0
4		0·176		0		0	
5		0·196		0		0	
6		0·19		0		0	

Deaths were recorded over the ensuing fourteen days. The vaccinated guinea pigs and six non-vaccinated controls were challenged eight weeks after vaccination by the method of McDiarmid and Sutherland (1957). The vaccine was evaluated in the way described by these workers; for this purpose a group of six normal guinea pigs of similar age and weight were used.

Table 3 shows the protection afforded by S.19 vaccine to 100 $L.D._{50}$ calculated from the results shown in Table 1 and demonstrates the value of cortisone-treated mice for testing potency. Table 4 summarises the results on the guinea pig. The ratio of spleen-weight to bodyweight in the normal animal ranged from 0.176 to 0.245; there were two guinea pigs (B3 and B6) in the non-vaccinated challenged group, that fell within this range; there was also one vaccinated guinea pig, (A3) that fell outside this range. In respect of recovery of organisms from the spleen, the non-vaccinated challenged guinea pigs yielded counts varying from 5 to 200 organisms, but one vaccinated animal (A.10) also yielded 200 organisms. It will be noted that this was not the guinea pig with the highest spleen-weight to body-weight ratio. Agglutination titres ranged from 40 to 320 in the non-vaccinated and from 0 to 40 in the vaccinated group.

DISCUSSION

The results of this study demonstrate that the mouse can be used for testing *Br. abortus* (S.19) vaccine. It has the advantages over the guinea pig that it is less expensive, that the test takes less time (five weeks compared with 16 weeks), that the results are easier to interpret and that there is less risk to those carrying out the test.

The interpretation of results on the guinea pig presents considerable difficulty when agglutination titres and spleen-weight to body-weight ratios are taken as criteria of infection; the dependence on arbitrary limits makes variations in assessment almost unavoidable. For example, in our results an agglutination titre of less than 40 could be taken as indicative of protection; similarly, a spleen-weight to body-weight ratio of 0·25 could also suggest protection. The recovery of organisms from the spleen would appear to be a better criterion; it may be noted that McDiarmid and Sutherland (1957) were presumably of this opinion. The method is, however, laborious and time-consuming and associated with some risk to laboratory workers, since each individual spleen has to be removed and homogenised.

CONCLUSIONS

The susceptibility of mice to *Brucella abortus,* S.544 was increased at least one thousand-fold after their treament with cortisone. A description is given of methods employing mice for the testing of S.19 vaccine.

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THE EFFECT OF STREPTOMYCIN ON THE SURVIVAL OF *VIBRIO FETUS* IN SEMEN AND OTHER DILUENTS STORED AT 5° AND −79°C.

By

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INTRODUCTION

The addition of anti-bacterial agents to semen diluents is widely used in routine artificial insemination practice. Different anti-bacterial agents, singly or in combination and at different concentration levels, have been studied with respect to their effect on viability and metabolism of spermatozoa and on bacterial counts. This work has been reviewed by Easterbrooks (1951) and Willett and Ohms (1955).

Since 1951, it has been generally recognised that *Vibrio fetus* causes a temporary infertility in cows and that the disease can also be spread through the use of artificial insemination using the semen of carrier bulls (Terpstra and Eisma, 1951; Lawson and MacKinnon, 1952; McEntee, Hughes and Gilman, 1954a). The addition of antibiotics to semen diluents has, therefore, been directed not so much against the control of bacterial contaminants normally present in bull semen or their action on spermatozoan metabolism as against their action on specific organisms which may be present in bull semen and can cause infertility. Included in this group of specific organisms is *V.fetus*.

Increased conception rates following the dilution of semen from *V.fetus* infected A.I. bulls in diluents containing antibiotics were reported by Adler and Rasbech (1952, 1953) and Willett, Ohms, Frank, Bryner and Bartlett (1955). Frank and Bryner (1953), McEntee, Hughes and Gilman (1954b), Hughes (1956) and Adler (1957) reported that heifers could not be infected when inseminated with *V. fetus* infected semen diluted in egg yolk—citrate buffer containing antibiotics, but this was not confirmed by Lawson and MacKinnon (1952) and Lawson (1954).

In routine artificial insemination practice, the semen, after dilution in a buffer containing antibiotics, is stored at 5°C. until used. Since pathogenic bacteria do not multiply at 5°C. and since penicillin especially does not act on non-multiplying organisms, considerable controversy has existed as to whether *V.fetus* is killed in vitro at 5°C. or at body temperature after insemination. The recovery of *V. fetus* from the standard egg yolk–citrate buffer containing various antibiotics and stored at 5°C. has been reported by many workers (Plastridge and Easterbrooks, 1952, Albertsen, 1952, 1953, 1957; McPherson and Fish, 1954; Orthey and Gilman, 1954a; Lenz, 1956). However, Orthey and Gilman (1954b) reported that they could not recover *V.fetus* from egg yolk citrate buffer containing both penicillin and streptomycin after storage for at least six hours at 5°C. The survival of *V.fetus* in sterile skim milk diluent containing various antibiotics has been reported by Dilliello, Poelma and Faber (1957).

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The purpose of the present investigations was to study further whether *V.fetus* could survive in semen diluents and culture media containing antibiotics after storage at various temperatures; and whether *V.fetus* infected semen, diluted in various semen diluents containing streptomycin (500 μg/ml. diluted semen) could still infect heifers after storage for varying times at 5°C. and −79°C.

MATERIALS AND METHODS

Egg yolk-citrate diluent was prepared by mixing equal volumes of egg yolk and 3·6 per cent (w./v.) solution of sodium citrate. Skim milk diluent was prepared by reconstituting, at 50°C., skim milk powder to make a 9 per cent (w./v.) solution. This was heated to 92°C. for 10 minutes and cooled before using. Other diluents used were N sterile saline, Brewer's medium and nutrient broth. Streptomycin (calcium chloride complex), chlortetracycline (aureomycin) and chloramphenicol, obtained as powders, were dissolved in sterile, distilled water and were added to the various diluents to give the required final concentrations (Table 1). Sterile distilled water only was added to control diluents not containing antibiotics.

The strains of *V.fetus* were grown on thio-blood agar (Morgan, 1957) and, after three days of incubation at 37°C. in 10 per cent CO_2, the growth was suspended in nutrient broth. 0·25 ml. volumes of this dense suspension were added to 10 ml. of the diluents and stored at either 5° or 37°C. Periodically, 2·0 ml. volumes of the antibiotic-containing diluents were centrifuged, the centrifugate washed and then cultured on thio-blood agar. In the case of tubes not containing antibiotics, cultures were made directly, although occasionally their contents were centrifuged and treated in exactly the same way as the antibiotic-containing mixtures.

For studying the survival of *V.fetus* at −79°C., the technique used for the preservation of bull semen described by Bruce (1956) was used, except that a suspension of *V.fetus* was used instead of semen. The survival of *V.fetus* was studied at −79°C. in egg yolk-citrate diluent containing the following ingredients (a) 10 per cent glycerol and 500μg./ml. streptomycin, (GS); (b) 500μg./ml. streptomycin (no glycerol), (S); (c) 10 per cent glycerol (no streptomycin), (G); (d) no glycerol or streptomycin, (B). At varying times, ampoules were thawed at room temerature and, initially, the contents were cultured directly on thio-blood agar. Later, cultural examinations were made after centrifugation and washing to avoid carrying over of antibiotic on to the plates.

All plates were incubated at 37°C. for seven days and any vibrios found were subcultured and examined for catalase and hydrogen sulphide production as previously described (Morgan, 1957). Occasionally, cultures so obtained were also compared with the original cultures for sensitivity to the antibiotics.

Heifers used for the infectivity experiments were shown to be free of *V.fetus* infection by repeated cultural examination of vaginal mucus and/or uterine biopsy samples. In the latter part of the work, negative heifers that had been previously used for diagnosis (Morgan, 1956; Morgan, Melrose and Stewart, 1958) were employed. Semen from known infected bulls was diluted in the various diluents.

Egg yolk-citrate buffer. Semen from infected bull S12 was collected and diluted in egg yolk-citrate diluent at room temperature as follows:—

Dilution rate	*Concentration of streptomycin* (μg./ml. diluted semen)
Undiluted	0
1/50	500
1/50	500

All the semen samples were stored at 5°C. for two hours. Heifers in oestrus were then inseminated intracervically with 1·0 ml. volumes of the semen, as shown in Table 3.

Skim milk diluent. Semen from infected bull ED was diluted in reconstituted skim milk diluent at 20°C. as follows:

Dilution rate	*Concentration of streptomycin* (μg./ml. diluted semen)
Undiluted	0
1/5	0
1/5	500
1/50	0
1/50	500

After collection and dilution, the semen was kept at 5°C. After 2½ and 6 hours' storage, heifers in oestrus were inseminated intracervically with 1·0 ml. of the semen samples, as shown in Table 4.

Deep frozen semen. A batch of deep-frozen semen was received at the laboratory in a flask of solid CO_2. The semen had been diluted 1/20 in egg yolk-citrate buffer and contained 500 μg. streptomycin and 500 μ. penicillin per ml. diluted semen. The contents of the vials, which had been stored for approximately one year at −79°C., were thawed at room temperature and used for inseminating heifers in oestrus, as shown in Table 5. In all cases, anterior vaginal mucus was collected from all the heifers after insemination and cultured in the usual way.

RESULTS

Survival of V. fetus *in Various Diluents with Antibiotics.*

The results obtained are recorded in the following tables. In the case of the egg-yolk citrate and milk diluents, the experiments were repeated on at least six separate occasions with four strains of *V. fetus*. From Table 1 it can be seen that *V. fetus* could be recovered from egg-yolk citrate buffer containing 500 μg/ml. streptomycin when stored at 4°C. after 72 hours' storage. At 37°C. in the presence of streptomycin, the organism could not be recovered except on one occasion when it was recovered after 24 and 48 hours' storage. Without antibiotics, *V. fetus* survived well in egg-yolk citrate buffer especially at 4°C.; at 37°C., growth was often overwhelmed by the growth of contaminants especially sporing rods. Similar results were obtained by using dihydrostreptomycin. At 4°C., *V. fetus* could be recovered after 72 hours' storage in egg yolk-buffer containing chlortetracycline (100 μg/ml.) and after 24 hours in buffer containing 250 μg/ml. chloramphenicol.

In reconstituted skim milk diluent, containing streptomycin

(500 μg/ml.) *V.fetus* could not be recovered at 37°C. nor at 4°C. except on one occasion when a few colonies were recovered after 24 and 48 hours. Chlortetracycline (100 μg/ml.) had no effect on *V.fetus* in milk diluent either at 4°C. or 37°C. and the organism could be recovered after 24 hours at 4°C. in the presence of 250 μg/ml. chloramphenicol.

TABLE 1

SURVIVAL OF *V.FETUS* IN VARIOUS DILUENTS IN THE PRESENCE OF ANTIBIOTICS*

Diluent	*Antibiotic*	*Conc.* (μg/ml)	*Temperature of storage*	*Viability after*			
				4 hrs.	*24 hrs.*	*48 hrs.*	*72 hrs.*
Egg-yolk citrate buffer	Streptomycin	500	37°C.	—	(—)	(—)	—
		500	4°C.	+	+	+	+
		0	37°C.	+	+	(+)	(+)
		0	4°C.	+	+	+	+
	Chlortetra-cycline	100	4°C.	+	+	+	+
	Chloramphenicol	250	4°C.	+	+	—	—
Reconstituted Skim milk diluent	Streptomycin	500	37°C.	—	—	—	—
		500	4°C.	—	(—)	(—)	—
		0	37°C.	+	+	(+)	—
		0	4°C.	+	+	(+)	(+)
	Chlortetra-cycline	100	37°C.	ND	+	+	—
			4°C.	+	+	+	+
	Chloramphenicol	250	4°C.	+	+	—	—
Lemco broth	Streptomycin	500	37°C.	—	—	—	—
		500	4°C.	—	—	—	—
		0	37°C.	+	+	+	+
		0	4°C.	+	+	+	+
Brewer's medium	Streptomycin	500	37°C.	—	—	—	—
		500	4°C.	—	—	—	—
		0	37°C.	+	+	+	+
		0	4°C.	+	+	+	+
N. saline	Streptomycin	500	37°C.	—	—	—	—
		500	4°C.	—	—	—	—
		0	37°C.	—	—	—	—
		0	4°C.	+	+	+	+

*With diluents containing antibiotics, cultural examinations were made after centrifugation and washing the deposit.
— = *V.fetus* not recovered
\+ = *V.fetus* recovered
(—) = *V.fetus* occasionally recovered
(+) = *V.fetus* usually recovered, negative results being mostly due to the overwhelming growth of contaminants.
N.D. = No observation made.

V.fetus could not be recovered from either lemco broth or Brewer's medium containing 500 μg/ml. streptomycin at either

4°C. or 37°C., but could be recovered in these media without antibiotic at both temperatures. The organism could not be recovered from saline containing streptomycin at either 4°C. or 37°C. It could not be recovered from saline stored at 37°C., but could be recovered at 4°C.

V. fetus could survive at −79°C. in egg-yolk citrate buffer either in the presence of 10 per cent glycerol or without glycerol (Table 2). However, in the presence of streptomycin, the organism could not be recovered by direct cultural examination on thio blood agar plates, but where the streptomycin was first removed by centrifugation followed by washing the deposit, *V. fetus* was also recovered from the egg-yolk citrate buffer containing 500 μg/ml. streptomycin with or without the presence of 10 per cent glycerol, after 180 days' storage at −79°C. Observations were not made beyond this period. However, it was noticed that plates inoculated from buffer containing glycerol, irrespective of the presence of streptomycin, gave more growth than those inoculated from buffer not containing glycerol indicating that the glycerol had a protective effect on the organism.

TABLE 2

SURVIVAL OF *V.FETUS* IN EGG-YOLK CITRATE BUFFER AT −79°C.

Reference (C)	*Length of storage (days)*						
	6	*12*	*20*	*40*	*59*	*160a*	*180a*
GS	—	—	—	—	—	+	+
S	—	—	—	—	—	+	+
G	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+
Experiment 2							
GS	No	—	—	—		+	+
S	experi-	—	—	—		+	+
G	ments	+	+	+		+	+
B	made	+	+	+		+	+

— = *V.fetus* not recovered
\+ = *V.fetus* recovered
a = Contents of vials centrifuged and washed before culturing. At the previous examinations, cultures were made directly on plates without prior centrifugation.
(C) Egg-yolk citrate diluent containing
GS plus 10% glycerol and 500 μg/ml. streptomycin.
S plus 500 μg./ml. streptomycin.
G plus 10% glycerol.
B no glycerol or streptomycin.

All the four strains used throughout this work were sensitive to 5 μg/ml. streptomycin. The one strain used for the chlortetracycline and chloramphenicol experiments was sensitive to 0·3125 μg/ml. and 125 μg/ml. respectively. Furthermore, strains isolated from buffer containing streptomycin were all examined for sensitivity to streptomycin to find out whether growth was caused by the emergence of

resistant strains. Growth was, in all cases, inhibited by 5 μg/ml. streptomycin showing that resistant strains did not arise.

TABLE 3

THE USE OF EGG-YOLK CITRATE BUFFER

Heifer No.	*Dilution of semen*	*Conc. of streptomycin μg/ml. diluted semen*	*Length of storage (hours) before insemination*	*Cultural results**	*Mucus agglutination results*
21	undiluted	0	2	+	+
19	1/5	500	2	+	+
20	1/5	500	2	—	—
41	1/5	500	2	+	+
32	1/50	500	2	—	—
33	1/50	500	2	+	+

* + = *V. fetus* recovered from oestrous mucus and uterine biopsy samples.
— = *V. fetus* not recovered.

Infectivity of V. fetus *Semen in Diluent containing 500 μg. Streptomycin at 5° and −79°C.*

The results obtained are summarized in Tables 3 to 5.

From Table 3 it can be seen that two of the three heifers inseminated with semen diluted 1:5 in egg-yolk citrate buffer containing 500 μg/ml. streptomycin and one of two heifers inseminated with semen diluted 1:50 plus 500 μg/ml. streptomycin became infected, as did the heifer inseminated with the undiluted semen. The organism was isolated from oestrous mucus and uterine biopsy samples; in all cases it was catalase positive and did not produce hydrogen sulphide. All the heifers that were culturally positive were also positive to the mucus agglutination test. In this experiment, the heifers were observed over a period of five months.

When reconstituted skim milk was used as diluent, the results obtained were quite different (Table 4). Semen diluted in skim milk diluent without streptomycin and stored for 2½ hours at 5°C. was still able to infect all the four heifers, both at the 1:5 and the 1:50 dilution rates. Under the same conditions of storage time and dilution but in the presence of 500 μg/ml. streptomycin, none of four heifers became infected, but catalase negative vibrios were isolated from two of these heifers.

When semen diluted 1:50 and stored for 6 hours at 5°C. was used for insemination, one of two heifers became infected when the diluent did not contain streptomycin and none of two heifers when the diluent contained streptomycin (500 μg/ml.) The heifer inseminated with the undiluted (control) semen became infected.

In the case of the deep-frozen semen, one heifer was inseminated with 3 ml. and another with 1 ml. of semen from different collections from the same bull; both heifers became infected.

TABLE 4

THE USE OF SKIM MILK DILUENT

Heifer No.	*Dilution of semen*	*Conc. of streptomycin μg/ml. diluted semen*	*Length of storage (hours) before insemination*	*Cultural results* [a]	*Mucus agglutination results*
118	1/5	0	$2\frac{1}{2}$	+	—
144	1/5	0	$2\frac{1}{2}$	+	—
104	1/5	500	$2\frac{1}{2}$	—	—
133	1/5	500	$2\frac{1}{2}$	—[b]	—
137	1/50	0	$2\frac{1}{2}$	+	—
114	1/50	0	$2\frac{1}{2}$	+	—
145	1/50	500	$2\frac{1}{2}$	—	—
110	1/50	500	$2\frac{1}{2}$	—[b]	—
140	1/50	0	6	+	—
117	1/50	0	6	—	—
105	1/50	500	6	—[b]	—
129	1/50	500	6	—	—
119	undiluted	0	$2\frac{1}{2}$	+	+

a { + = *V.fetus* recovered from anterior vaginal mucus.
  { — = *V.fetus* not recovered.
b = catalase negative vibrios recovered.

TABLE 5

USE OF DEEP-FROZEN SEMEN

Heifer	*Semen*	*Cultural examination* [a]
150	3 ampoules (3 ml.) deep-frozen semen	+
106	1 ampoule (1 ml.) deep-frozen semen	+

a = *V.fetus* recovered from anterior vaginal mucus.

DISCUSSION

The observations on the survival of *V.fetus* in semen and other diluents containing large quantities of various antibiotics revealed some interesting results. *V.fetus* could be recovered from egg-yolk citrate buffer containing 500 μg/ml. streptomycin when stored at 5°C. for 72 hours, but not when incubated at 37°C. Chlortetracycline and chloramphenicol were also ineffective at 5°C. The results obtained with streptomycin confirm those reported by Plastridge and Easterbrooks (1952), Albertsen (1952, 1953, 1957)

and Lenz (1956), but do not support Orthey and Gilman (1954a, 1954b), Hughes (1956) and Adler (1957). The recovery of *V.fetus* from egg-yolk citrate buffer with or without glycerol and in the presence or absence of streptomycin after storage at −79°C. also confirms the results of McPherson and Fish (1954). Albertsen (1952) suggested that the presence of lipids in egg-yolk protected the organism against the bactericidal action of streptomycin. However, the fact that streptomycin was bactericidal in egg-yolk citrate at 37°C. as reported in this work would suggest that either this is not the case or that the protective effect on the organism is only elicited at 5°C.

In reconstituted skim milk diluent, lemco broth, Brewer's medium and normal saline containing 500 μg/ml. streptomycin, *V.fetus* could not be recovered after incubation at either 5°C. or 37°C. It follows, therefore that in these diluents at this concentration, streptomycin is bactericidal against *V.fetus* at 5°C. The results obtained using reconstituted skim milk diluent confirm those reported by Dilliello *et al.* (1957) except that these authors noted a difference between the results obtained with streptomycin and dihydrostreptomycin. The unsatisfactory results obtained with chlortetracycline might be due to its rapid deterioration in vitro as reported by Bliss and Todd (1949).

Gunnison, Kunishige, Coleman and Jawetz (1955) studied the effect in vitro of various antibiotics on bacteria not actively multiplying. They found that neomycin, polymixin and to a lesser extent, streptomycin, were far more effective in water than in broth at 37°C. At 5°C., the bactericidal effect of the antibiotics varied widely with the test micro-organism. The minimum bactericidal dose of streptomycin against *Escherichia coli* in water at 4°C. and 37°C. was 5 μg. in each case; in broth, the respective values were 500 and 100 μg. Although penicillin is most effective against multiplying organisms (Hobby, Meyer and Chaffer, 1942; Lee, Foley and Epstein, 1944; Gunnison *et al.*, 1955) no hard and fast rules can be laid down for streptomycin. The results of Gunnison *et al.* (1955) show that streptomycin is bactericidal against some species both at 37°C. in water and at 4°C.

In the present work, the minimum bactericidal doses of streptomycin, as defined by Gunnison *et al.* (1955), for *V.fetus* at various temperatures and media were not assessed, but the results show that in the presence of large concentrations streptomycin is bactericidal in milk diluent, broth, Brewer's medium and saline at 4°C. but not in egg-yolk citrate buffer. It has been suggested following the work of Gunnison *et al.* (1955) and Albertsen (1952) that antibiotics accelerate the death rate of the bacteria studied when stored at 4°C. This point was not investigated in these studies, but in nearly all cases *V.fetus* could be recovered from all the diluents not containing antibiotics after storage at 4°C. The only exception was the rapid death of the organism in saline at 37°C.

The in vitro results on the survival of *V.fetus* in semen diluents containing streptomycin were confirmed by the in vivo experiments using heifers in oestrus. It was shown that infected semen diluted 1:5 and 1:50 in egg-yolk citrate buffer containing 500 μg. streptomycin per ml. diluted semen could still infect two of three and one of two heifers respectively after two hours' storage at 5°C. These results do not accord with those reported by Frank and Bryner (1953), McEntee *et al.* (1954b), Hughes (1956) and Adler (1957), but are in agreement with those reported in this country by Lawson and MacKinnon (1952) and Lawson (1954). Plastridge and Easterbrooks (1952) and Albertsen (1957), who observed that *V.fetus* could survive exposure to streptomycin at 5°C., suggested that the antibiotic would be effective at body temperature after insemination. This view is not borne out by the present experiments. Lawson (1954) actually allowed the diluted semen-antibiotic mixture to stand at room temperature for two hours before it was stored at 4°C. prior to insemination. Even under these conditions, a proportion of the inseminated heifers became infected. This would discount the recommendation made by Albertsen (1957) that semen diluted in antibiotic containing diluent should be kept at 30°C. for two hours prior to storage at 5°C. in order to allow the antibiotics to destroy the organisms before storage and insemination.

Using reconstituted skim milk powder as diluent, the results showed that streptomycin was effective in preventing all the heifers from becoming infected irrespective of the dilution rate and the storage time. That this was not due to either a dilution effect or the normal destruction of the organisms at refrigeration temperature was shown by the fact that all the heifers inseminated with semen diluted 1:5 and 1:50 with no streptomycin and stored for 2½ hours became infected and one of two heifers inseminated with semen diluted 1:50 and stored for six hours. This is the first report of the effectiveness of streptomycin in milk diluents against *V.fetus* under actual artificial insemination procedures.

These observations were supported by Melrose, Stewart and Bruce (1958). In a split sample field fertility trial involving 17 bulls at the Reading Cattle Breeding Centre and 10,000 cows, a significantly greater conception rate was obtained with skim milk diluent than with egg-yolk citrate diluent. Both diluents contained 500 μg. streptomycin per ml. diluted semen. The increased conception rates were confined to 6 of the 17 bulls. It was later shown that 2 of these 6 bulls were infected with *V.fetus*. No explanation could be found to account for the increased conception rates obtained with the other four bulls.

The fact that two heifers became infected after insemination with semen diluted approximately 1:20 in egg yolk citrate buffer containing 10 per cent glycerol and 500 u. penicillin and 500 μg. streptomycin per ml. after about a year's storage at −79°C. confirms the laboratory findings quoted earlier. This is the first occasion on

which it has been proved that deep-frozen semen is still capable of causing infection.

CONCLUSION

V.fetus survived storage for 3 days in egg-yolk citrate buffer containing 500 μg/ml. streptomycin at 5°C. *V.fetus* was also recovered from egg-yolk citrate buffer containing 500 μg/ml. streptomycin after 180 days' storage at −79°C. However, in reconstituted skim milk diluent, broth, Brewer's medium and saline containing 500 μg/ml. streptomycin, it could not be recovered even after 4 hours' storage at 5°C.

Infected semen diluted 1/5 and 1/50 in egg-yolk citrate buffer containing 500 μg. streptomycin per ml. diluted semen was capable of infecting heifers after 2 hours' storage at 5°C.

Infected semen diluted 1/5 and 1/50 in reconstituted skim milk diluent containing 500 μg. streptomycin per ml. diluted semen was not capable of infecting heifers after 2 hours' or 6 hours' storage at 5°C. Semen similarly diluted but with no streptomycin was capable of infecting heifers under similar conditions.

Infected semen diluted in egg yolk citrate buffer containing 500 u/ml. penicillin, 500 μg/ml. streptomycin and 10 per cent glycerol was able to infect heifers after approximately a year's storage at −79°C.

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FURTHER STUDIES ON SCRAPIE

By

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INTRODUCTION

A review of the contradictory evidence as to the nature of scrapie disease of sheep has recently been given by Stamp (1958). In brief, a number of workers claim to have transmitted the condition by injecting into healthy sheep the brain and spinal cord of animals affected with scrapie (Cuillé and Chelle, 1936; Gordon, 1946; Wilson, Anderson and Smith, 1950; Wilson, 1952). Wilson and his co-workers also transmitted the disease in series from sheep to sheep over nine passages and confirmed the earlier finding of Cuillé and Chelle that the transmissible agent could be filtered through virus filters. They interpret their results to mean that scrapie is due to a virus infection of the central nervous system. Further experiments carried out by Wilson (1954) have shown that the transmissible agent is unusually resistant and that amongst other things it can survive boiling at 100°C. for thirty minutes. Gordon (1946) and Greig (1950) had already shown that the agent could resist a concentration of formalin of 0·35 per cent over a period of at least three months. Recently, however, Bosanquet, Daniel and Parry (1956) and Parry (1957) have suggested that scrapie is an inherited defect and is part of a more widely occurring primary myopathy of sheep. They consider that the interpretation of the results of the above inoculation experiments purporting to establish scrapie as an infection of the central nervous system is open to doubt. In their opinion there are good grounds for suspecting that the so-called "normal" sheep used for transmission work were not free from the myopathic trait in that they were mainly of a breed well known to suffer from scrapie. A leading article (Anon, 1956) states that the data hitherto thought to establish scrapie as an infectious disease needs reappraisal.

The present paper is concerned with further transmission experiments.

MATERIALS AND METHODS

Experimental animals. In 1955 six hundred and twenty-one Cheviot wether hoggs, aged about six months, were purchased from several farms thought to be free from scrapie. A few Blackface hoggs of similar age were also bought. In 1956 a further two hundred and four Cheviot hoggs were purchased. In the spring of 1956 fourteen Cheviot day-old lambs bred from old Cheviot ewes purchased two years previously and then maintained at the Institute were used in one experiment. This was followed by the purchase in the autumn of 1956 of eighty-seven cast Cheviot ewes, ten Blackface cast ewes and several rams which produced for experimental use one hundred and twelve Cheviot lambs and eight Blackface lambs in the spring of 1957. The sheep used in the experiments were randomly allocated. Material for inoculation was stored at −40°C.

Scrapie sheep brain pools. In 1955 brains from twelve experimental cases of scrapie which had been stored for periods of one to five months were homogenised, pooled and distributed into cartons in 100 g. quantities (scrapie sheep brain pool/1955). In 1956 a pool was prepared in a similar way using brains from twenty-four experimental cases of scrapie which had been produced in Cheviot hoggs (1955) as a result of intracerebral inoculation with S.S.B.P./1955 (scrapie sheep brain pool/1956). A further scrapie sheep brain pool was prepared in 1956 made up from the brains and spleens of sheep affected with natural scrapie (natural scrapie sheep tissue pool/1956).

Normal sheep brain pool. Brains from twenty of the healthy Cheviot hoggs (1955) taken by random sample were obtained immediately after slaughter and one half of each brain was homogenised, pooled and stored in 100 g. quantities (normal sheep brain pool/1955). The remaining half of each brain was taken for histological examination and subsequently showed no lesions specific for scrapie (Zlotnik and Rennie, 1958).

Other tissues and body fluids. On occasion, homogenised spleen, lymph gland and skin tissues as well as cerebrospinal fluid taken from experimental scrapied sheep were used. With the exception of cerebrospinal fluid which was inoculated undiluted, all material for inoculation was prepared by making a 1/10 saline suspension of tissue which was subsequently spun at 3,000 r.p.m. for one hour. One ml. of the supernatant fluid was used for the intracerebral route and 5 ml. for the subcutaneous route of infection.

Diagnosis of scrapie was based on advanced clinical signs such as severe rubbing, inco-ordination of movement and emaciation, but confirmation of diagnosis by histological examination of the brain (Zlotnik, 1958) was carried out in every case.

Strict precautions were taken to avoid cross-contamination of the various inocula; separate groups of instruments and glassware newly purchased were used for the different experimental groups. The only method of sterilisation used was that of autoclaving at 15 lb. pressure for thirty minutes, a measure known to destroy the transmissibility of scrapie material.

No attempt was made to isolate any group of sheep: most animals were housed together in a large Dutch barn and fed on sheep nuts, hay and water. This type of housing was necessary since isolation facilities were not available for the large number of sheep used on experiment. However, as the experimental sheep were discarded twelve months after inoculation the above method of keeping the experimental animals seemed acceptable for there is considerable evidence that uninoculated controls living alongside numerous cases of scrapie for longer periods of time (up to $3\frac{1}{2}$ years) do not develop the disease. The discarding of sheep twelve months after inoculation, that is when they are less than twenty months old, prevents any confusion with natural scrapie since this rarely occurs in sheep younger than this.

RESULTS

Scrapie Sheep Tissue Experiments

Inoculation of scrapie sheep brain pool/1955. This was a control experiment to test the capacity of the straight pool material to

produce scrapie. At the same time comparisons between the susceptibility of Cheviot and Blackface sheep and between intracerebral and subcutaneous routes of inoculation were made. Of the thirty 1955 Cheviot hoggs inoculated intracerebrally ten developed scrapie within 5 to 5½ months; of the thirty 1955 Blackface hoggs inoculated intracerebrally seven developed scrapie within five months and of the thirty 1955 Cheviot hoggs inoculated subcutaneously nine developed scrapie within 5 to 8 months. Pooled brain collected from a number of the Cheviot sheep which developed scrapie following subcutaneous inoculation with sheep brain pool/1955 was inoculated intracerebrally into ten of the 1956 Cheviot hoggs. Of these five developed scrapie within five to eight months.

TABLE 1

EXPERIMENTAL TRANSMISSION WITH BRAIN

Inoculum	*Number and type of sheep inoculated*	*Route of inoculation*	*Number of sheep developing scrapie*	*Incubation period in months*
Scrapie sheep brain pool (1955)	30 Cheviot hoggs	Intracerebral	10	5–5½
ditto	30 Blackface hoggs	Intracerebral	7	5
ditto	30 Cheviot hoggs	Subcutaneous	9	5–8
Brains of sheep developing scrapie as a result of subcutaneous inoculation of scrapie sheep brain pool (1955)	10 Cheviot hoggs	Intracerebral	5	5–8
Scrapie sheep brain pool (1955)	14 new-born Cheviot lambs	Intracerebral	11	3½–9
Scrapie sheep brain pool (1956)	15 new-born Cheviot lambs	Intracerebral	6	5–6
Scrapie sheep brain pool (1956)	6 new-born Blackface lambs	Intracerebral	0	—
Scrapie sheep brain pool (1955) with adjuvant	10 Cheviot hoggs (6 survived after 2 months)	Intramuscular	3	5–7
Scrapie sheep brain pool (1955) with killed louping ill virus	30 Cheviot hoggs	Subcutaneous	2	5–6
Scrapie sheep brain pool (1955) (control to above)	30 Cheviot hoggs	Subcutaneous	5	5–8
Scrapie sheep brain pool (1956)	10 new-born Cheviot lambs	By mouth (5 ml.)	0	—
Normal sheep brain pool (1955)	20 Cheviot hoggs	Intracerebral	0	—
ditto	20 Blackface hoggs	ditto	0	—
Normal sheep brain pool (1955) with adjuvant	10 Cheviot hoggs (6 survived after 2 months)	Intramuscular	0	—

Inoculation of scrapie sheep brain pool into day-old lambs. Of fourteen 1956 Cheviot lambs inoculated intracerebrally within twelve hours of birth with scrapie sheep brain pool/1955, eleven developed typical symptoms of scrapie in $3\frac{1}{2}$ to 9 months. The other three lambs remained healthy. Of fifteen 1957 Cheviot lambs inoculated intracerebrally within twelve hours of birth with scrapie sheep brain pool/1956 six developed scrapie in 5 to 6 months. Of six 1957 Blackface lambs inoculated intracerebrally as above none developed scrapie.

Inoculation of scrapie sheep brain pool/1955 plus adjuvant. Brain material from scrapie sheep brain pool/1955 was suspended in saline; 50 ml. of this fifty per cent. suspension along with 0·2 g. of *Mycobacterium phlei* and 200 ml. of Bayol and Falba (4 parts to 1 part) were atomixed and homogenised. Each of ten 1955 Cheviot hoggs was inoculated intramuscularly with 5 ml., 1·25 ml. into each limb. Eight out of the ten sheep developed experimental allergic encephalitis (E.A.E.) in 7, 12, 19, 34, 38, 45, 60 and 103 days after inoculation. Two cases of scrapie developed in the four sheep which survived the allergic encephalitis and one case in the two sheep which did not develop E.A.E.

Inoculation of scrapie sheep brain pool/1955 along with killed louping-ill virus. It was considered possible that inactivated louping-ill virus might assist the scrapie factor to produce a higher incidence of experimental scrapie in inoculated sheep since it was known that one batch of louping-ill vaccine had caused scrapie to occur in vaccinated sheep (Gordon, 1946).

In consequence, twenty Cheviot sheep were each inoculated intracerebrally with 1 ml. of scrapie sheep brain pool/1955. Four months later half of these sheep were infected with louping-ill virus, their brains and spleens then being made into standard louping-ill vaccine, a product in which the louping-ill virus is inactivated by 0·3 per cent. formaldehyde for at least thirteen days at 4°C. The other half of the sheep were not injected with louping-ill virus but their brains and spleens were treated in the same way as those used for the vaccine. Each lot of material was inoculated subcutaneously into thirty 1955 Cheviot hoggs.

Only two sheep developed scrapie in the louping-ill vaccine group, one five months and one six months after inoculation. Of the thirty sheep inoculated with similar material less the inactivated louping-ill virus, five developed scrapie. The period in months after inoculation was 5, 6, 7 and 8 (two cases).

Transmission by spleen and lymph gland but not by skin. Six of the 1955 Cheviot hoggs, inoculated intracerebrally with scrapie sheep brain pool/1955 and which subsequently developed symptoms of scrapie, were used in the next experiment. Skin, which was taken from the four quarters and face, the spleen and the lymph glands (hepatic, mediastinal, parotid, prescapular and precrural) were

removed; the different types of tissues were pulped and stored at −40°C. as three separate groups. At the time of inoculation 5 g. of each tissue pool were mixed and made to a 1/10 dilution with normal saline. This material was centrifuged at 3,000 r.p.m. for one hour, the supernatant then being filtered through Kieselguhr and then through a 720 mμ gradocol membrane. One ml. of the filtrate was inoculated intracerebrally into each of ten 1955 hoggs. Six of these animals developed scrapie within 5 to 9 months. At a later date 5 ml. of a similar filtrate was inoculated subcutaneously into ten of the 1956 Cheviot hoggs and of these three developed scrapie in 8 to 9 months. Pooled brains taken from the six hoggs which supplied the skin, spleen and lymph glands when injected intracerebrally into ten 1955 Cheviot hoggs caused scrapie to develop in three of these sheep.

As the three tissues, skin, spleen and lymph glands, used as above had caused scrapie to appear in inoculated sheep each tissue was next tested individually by intracerebral inoculation of ten 1956 Cheviot hoggs. Three sheep inoculated with spleen developed scrapie in 5 to 7 months, two sheep inoculated with lymph gland developed the disease in 7 months while the sheep receiving skin remained healthy.

TABLE 2

EXPERIMENTAL TRANSMISSION WITH OTHER TISSUES

Inoculum	*Number and type of sheep inoculated*	*Route of inoculation*	*Number of sheep developing scrapie*	*Incubation period in months*
Skin, spleen and lymph glands from scrapie sheep	10 Cheviot hoggs	Intracerebral	6	5–9
ditto	10 Cheviot hoggs	Subcutaneous	3	8–9
Skin as above	10 Cheviot hoggs	Intracerebral	0	—
Lymph glands as above	10 Cheviot hoggs	ditto	2	7
Spleen as above	10 Cheviot hoggs	ditto	3	5–7
Cerebro-spinal fluid from sheep with experimental scrapie	10 Cheviot hoggs	Intracerebral	3	7–9
Natural scrapie tissue pool (1956)	19 new-born Cheviot lambs	Intracerebral and subcutaneous	7	5–6
Scrapie sheep brain pool (1955) after five two month blind passages in sheep	20 Cheviot hoggs	Intracerebral	3	5–8

Transmission by cerebrospinal fluid. Quantities of cerebrospinal fluid taken from five cases of experimental scrapie (1955 Cheviot hoggs inoculated with scrapie sheep brain pool/1955) were stored individually at −35°C. for several months. Just before use 2 ml.

of each sample were mixed and 1 ml. of this pool was inoculated intracerebrally into each of ten 1956 Cheviot hoggs. Three cases of scrapie developed within 7 to 9 months.

Recovery from tissues of natural cases. This pool was made up of the brains and spleens of two Cheviot, four Suffolk and three Swaledale sheep affected with natural scrapie. Halves of each organ were pooled together in an atomix while the other halves were kept separate; all the material was stored up to the time of use at −40°C. Of nineteen 1957 Cheviot day-old lambs inoculated both intracerebrally and subcutaneously with the pooled material seven developed scrapie in 5 to 6 months.

Transmission from parent to offspring. The brains and spleens of two Cheviot female lambs and three Suffolk wedder lambs born from parents affected with naturally occurring scrapie were pulped individually in an atomix. Half of each tissue pulp was then mixed to form a pool, of which 20 g. were made into a 1/10 suspension in saline. No cases of scrapie developed in the thirty 1956 Cheviot hoggs of which ten had been inoculated intracerebrally, ten subcutaneously and ten both intracerebrally and subcutaneously.

Transmission by mouth. A 5 ml. dose of the scrapie sheep brain pool/1956 was given to each of ten 1957 day-old Cheviot lambs before they sucked colostrum. No cases of scrapie developed in the seven lambs which survived: three lambs died within a few days of birth.

Rapid blind passage in sheep. A saline suspension of scrapie sheep brain pool/1955 was prepared in the usual way and 1 ml. was inoculated intracerebrally into each of ten 1955 Cheviot hoggs. Two months after inoculation, and before any symptoms of scrapie had developed, these sheep were slaughtered and their brains, except for portions taken for histological examination, were pooled and inoculated intracerebrally to a further ten 1955 Cheviot hoggs. This process was repeated over a further four passages, the final passage however being made into twenty of the 1956 Cheviot hoggs. These were then allowed to live for twelve months after the inoculation or until scrapie developed within that period. Of these twenty sheep three developed scrapie within 5 to 8 months of inoculation.

Normal Sheep Brain Experiments

Inoculation of normal sheep brain pool/1955 into six-month-old hoggs. Twenty of the 1955 Cheviot hoggs and twenty Blackface sheep were inoculated intracerebrally with 1 ml. of normal sheep brain pool/1955. No cases of scrapie developed.

Inoculation of normal sheep brain pool/1955 with adjuvant. Ten 1955 Cheviot hoggs were inoculated intramuscularly with normal Cheviot sheep brain made up with adjuvant as previously described for scrapie brain. Eight of the ten sheep developed symptoms of allergic encephalitis 9, 10, 11, 15, 19, 32, 40 and 242 days after

inoculation. No cases of scrapie developed in the four sheep which survived the allergic encephalitis or in the two sheep which remained free of E.A.E.

Characteristics of the Causal Factor

Resistance to heat. A 1/10 suspension of scrapie sheep brain pool/1955 in saline was prepared and centrifuged at 2,000 r.p.m. for 10 minutes.

Eighty ml. of the supernatant fluid were placed in the bottom of a 150 ml. flask (care being taken not to touch the neck with the pipette) which was then stoppered so that the whole flask could be totally immersed in a water-bath heated at 100°C., the immersed flask being shaken several times during the process. By this method the whole of the scrapie brain material was kept at 99·5°C. for thirty minutes. After cooling, the material was centrifuged at 2,000 r.p.m. for ten minutes, 1 ml. of the supernatant being inoculated intracerebrally into each of twenty 1955 Cheviots. Of the sixteen sheep surviving the inoculum, two developed scrapie seven months after injection.

TABLE 3

CHARACTERISTICS OF THE SCRAPIE PRODUCING FACTOR

Inoculum	*Number and type of sheep inoculated*	*Route of inoculation*	*Number of sheep developing scrapie*	*Incubation period in months*
Scrapie sheep brain pool (1955) heated to 99·5°C. for 30 mins. with intermittent shaking	20 Cheviot hoggs (16 survived after 2 months)	Intracerebral	2	7
Scrapie sheep brain pool (1956) heated to 99·5°C. for 30 mins. with constant shaking	15 new-born Cheviot lambs	Intracerebral	3	8
Scrapie sheep brain pool (1956) heated to 99·5°C. for 8 hours with constant shaking	21 new-born Cheviot lambs	Intracerebral	1	8
Brains of sheep developing scrapie following inoculation with above scrapie sheep brain pool (1955) heated 30 minutes	20 Cheviot hoggs	Intracerebral	6	5–6
Scrapie sheep brain pool (1955) exposed to acetylethyleneimine (Weston Hurst)	20 Cheviot hoggs	Intracerebral	3	6–7

A second batch was subjected to similar treatment except that the material, which was prepared from scrapie sheep brain pool/1956, was constantly stirred during the heat treatment. Fifteen

1956 Cheviot day-old lambs were inoculated intracerebrally: three lambs developed scrapie within 8 months.

A third batch also from the 1956 pool was held and constantly stirred at a temperature of 99·5°C. for eight hours, and was then inoculated intracerebrally into twenty-one 1957 Cheviot day-old lambs: one lamb developed scrapie within 8 months.

Serial transmission by heated scrapie brain. Brains from the sheep developing scrapie after receiving heated scrapie brain kept at at 99·5°C. for 30 minutes with intermittent shaking, were pooled and prepared for inoculation. Of twenty 1956 Cheviot hoggs inoculated intra-cerebrally six developed scrapie in 5 to 6 months and of twenty inoculated subcutaneously nine developed scrapie in 5 to 8 months.

Resistance of the scrapie factor to acetylethyleneimine. A 1/10 suspension of scrapie sheep brain pool/1955 was exposed to the action of acetylethyleneimine for a length of time exceeding by fifty per cent. that necessary to inactivate completely the most resistant of twelve viruses tested (eleven animal viruses and the virus of tobacco mosaic). The treated brain suspension was then lyophilised until just before use when it was reconstituted with distilled water. Twenty 1955 Cheviot hoggs were inoculated intracerebrally with this material and three developed scrapie within 6 to 7 months.

Survival in the cold. It has already been shown that the scrapie factor remains active in the dried state at 0° to 4°C. for long periods of time (Wilson *et al*, 1950) and the present work confirms this finding. The factor also remains active in frozen fresh tissues for several years when these are stored at —40°C.

Filtration. Wilson (1954) has shown that the scrapie factor passed a 400 mμ gradocol membrane but behaviour during passage through smaller membranes has not been investigated. A 1/10 saline suspension of scrapie sheep brain pool/1955 was centrifuged at 3,000 r.p.m. for 10 minutes. Thirty ml. of the supernatant were filtered through Kieselguhr and then successfully through a series of gradocol membranes of decreasing pore diameter, i.e. 1,240 mμ, 630 mμ, 400 mμ, 220 mμ, 110 mμ, 47 mμ and 27 mμ. The final filtrate passing through the 27 mμ membrane was inoculated intracerebrally into twenty 1955 Cheviot hoggs. Since a large number of animals would have been required, intermediate filtrates were not tested. The louping-ill virus preparation did not pass through the 27 mμ filter. The presence or absence of louping-ill virus was determined by the intracranial inoculation of mice. No cases of scrapie developed.

It was considered possible that the scrapie agent factor which survives a temperature of 99·5°C. for 30 minutes might be smaller than or chemically altered from the agent present in unheated material. For this reason the above filtration experiment was repeated but using the 30-minute-heated scrapie sheep brain pool/1955 already described. One ml. of the 27 mμ filtrate was inocu-

lated intracerebrally into twenty of the 1955 Cheviot hoggs. No cases of scrapie developed.

TABLE 4

DILUTIONS OF SCRAPIE BRAIN

Inoculum	*No. of sheep used*	*No. of cases of scrapie*	*Incubation*
10^{-1}	15	6	5 to 6 mths.
10^{-3}	10	4	ditto
10^{-5}	10	1	6 mths.
10^{-7}	10	0	—
10^{-9}	10	0	—

Dilution. Scrapie sheep brain pool/1956 was diluted to 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} with normal saline, each dilution being inoculated intracerebrally into groups of 1957 Cheviot hoggs. The resulting cases of scrapie are shown in Table 4.

DISCUSSION

The results of the above experiments show quite clearly that scrapie disease of sheep can be reproduced in Cheviot and Blackface hoggs by the inoculation of brain, spleen and lymph gland from sheep affected with either natural or experimental scrapie. In addition, it was found that Cheviot lambs inoculated intracerebrally within 24 hours of birth with scrapie brain developed symptoms of scrapie before they were 9 months old, that is at an age when natural scrapie is never seen. It was further found that spleen, lymph gland and cerebrospinal fluid taken from sheep affected with scrapie when inoculated into Cheviot hoggs also produced the disease. On the other hand, Cheviot hoggs, similar to those used for the positive transfer, inoculated with pooled brains (with and without adjuvant) taken from twenty of their normal companions failed to develop the disease within 12 months of being inoculated.

It might of course be thought that by chance none of the brains of the twenty clinically normal sheep used as inoculum were carrying a possible scrapie trait (Bosanquet, Daniel and Parry, 1956), whereas some of their companions used in other experiments were; this might well be so since the incidence of experimentally-induced scrapie in Cheviot sheep is never very high. However, a survey of the results obtained in the present experiment shows that experimentally-induced scrapie, in Cheviot hoggs and ewes inoculated either by the subcutaneous or intracerebral route with

untreated scrapie tissues, appeared in about one third of the animals. (By untreated scrapie tissues is meant tissues taken from scrapie-affected sheep and not subjected to heat or formaldehyde.) This figure is based on 135 inoculated sheep, but a similar incidence was also obtained on a larger number, 362 sheep, when the results of Wilson (1955) were taken into account. Further, it can be seen that the incidence of induced scrapie in the various groups is no more divergent than would be expected in groups randomly drawn from a population of experimental sheep e.g. X^2 8·93 Df = 9 P = 0·45. From these results, based on the method of Snedecor and Irwin (1933), it would seem that the likelihood of taking a sample of twenty brains free from a possible scrapie trait was small and, indeed, less than one chance in a thousand. In addition, the results of the dilution experiment suggest that the factor, if present, should not have been diluted out.

These findings therefore confirm and amplify the results of previous workers (Cuillé and Chelle, 1936; Gordon, 1946; Wilson, Anderson and Smith, 1950; Wilson, 1952) and suggest that the homogenised tissues of scrapie-diseased sheep contain some transmissible scrapie-producing factor not present in similarly prepared tissues of a sample of clinically normal sheep of the same breed and age. In addition, since tissues other than brain such as lymph glands, spleen and cerebrospinal fluid have now been shown to set up the experimental disease it cannot be merely a form of allergic encephalitis, a theory which has been postulated on a number of occasions. Further, as the scrapie factor has been successfully passaged blindly at two-monthly intervals through seven groups of Cheviot sheep it would appear that the agent is in fact replicating, for it would seem unlikely that at the end of this experiment the original inoculum could have been present in the sheep brains in sufficient quantity to cause scrapie to occur. Theoretically, the concentration of original brain present in the final passage is approximately 10^{-18} if the volumes of the inocula are taken into account in assessing this figure. It is of course possible that one or more of the sheep used in the later stages of this passage experiment were accidentally carrying the scrapie factor without showing clinical signs and that the scrapie arising at the end of the experiment was due to this latent factor rather than to a replication of the original agent, but this possibility, having already been experimentally investigated with negative results, seems unlikely.

There are a number of other points regarding the transmission of the disease which are of interest. Scottish Blackface sheep which are thought to be free of the natural disease are susceptible to the experimental disease with much the same incidence and incubation period as Cheviots. The susceptibility of the Blackface was clearly shown when scrapie was accidentally transmitted in louping-ill vaccine (Gordon, 1946; Greig, 1950) although the incubation period at that time was much longer. That the shorter incubation

period in the present experiments is not necessarily due to "laboratory adaptation" of the factor is however clearly shown since a new field agent has been isolated which in the first passage experiment produced cases of scrapie within 5 to 6 months of inoculation. Although scrapie can be readily produced experimentally by inoculation, the feeding of known scrapie material to newborn lambs did not produce the disease, but the number of animals used was probably too small for this observation to be of value.

The results of the dilution experiments were consistent with what one might expect with a biologically active particulate agent. The two negative results obtained with the higher dilutions of inoculum would appear to be significant and suggest that the active agent was diluted out between 10^{-5} and 10^{-9}.

The filtration experiment has shown that the active agent did not pass through a 27 mμ gradocol membrane capable of retaining louping-ill virus (Elford and Galloway, 1933). Wilson *et al.* (1950) had previously shown that the unheated agent would pass a 410 mμ membrane. Data was not obtained to determine the maximum blocking pore size of a membrane or whether the reduction in activity was caused by adsorption of the factor to the membrane surfaces.

As the experiments of Wilson (1954) have shown that the scrapie agent is resistant to heat in that it still produces scrapie after being boiled at 100°C. for thirty minutes, and since such a finding is incompatible with the present conception of a virus agent, it appeared essential to confirm and extend Wilson's findings. The results obtained do in fact confirm that the scrapie agent will without doubt withstand a temperature of 99·5°C., not only for thirty minutes but also for eight hours. The fact that the brains from the sheep developing scrapie following the inoculation of boiled material were themselves capable of producing scrapie when injected into further sheep, either by the intracerebral or subcutaneous route, indicates that the action of the boiled material is not simply that of a toxin. Evidence as to whether the heated fraction is self-replicating has however not been obtained. The present results also indicate that the scrapie agent which will withstand boiling is, like the unheated factor, unable to pass a 27 mμ A.P.D. membrane. In addition to being highly resistant to heat it was also found that the agent could withstand the action of acetylethyleneimine, a chemical substance which has a great affinity for nucleoprotein and which is known to abolish the infectivity of all viruses so far tested (Hurst, 1957).

It can be seen that the evidence as to the etiology of scrapie is difficult to interpret. On the one hand the results of transmission experiments suggest that scrapie is an infective disease caused by a particulate multiplying agent whereas the resistance of the transmissible factor to heat and chemical agents is such that it seems most unlikely that the factor could be nucleoprotein in nature. Since

on the above grounds it is difficult to accept that the positive results of transmission experiments are simply due to a virus in the inoculated material, other explanations have been considered. One of the most obvious is that the inoculated material contains two factors, one a true virus and the other a heat-resisting factor present in scrapie tissues which is capable of stimulating a latent scrapie virus in certain sheep. The fact that the inoculation by both the intracerebral and subcutaneous routes of boiled scrapie brain into day-old lambs gives rise to scrapie suggests however that either the theory is incorrect or that the latent virus is present in some sheep from the day of birth.

The interpretation of previous results of experimental transmission work which purport to establish scrapie as an infectious disease has been questioned by Bosanquet, Daniel and Parry (1956) on the grounds that the Cheviot sheep used could not be assumed to be free from all forms of scrapie. The validity of such a criticism is of course recognised but, nevertheless, there can now be no doubt that the inoculation of scrapie material in the numerous ways described, does produce the disease in sheep which otherwise would remain healthy. Further, it is possible to transmit the disease in series over a number of passages. Similar results cannot be brought about by inoculating materials from apparently normal sheep of the same breed as those supplying the scrapie tissues. Such findings make the acceptance of a hereditary hypothesis for scrapie (Bosanquet, Daniel and Parry, 1956) equally difficult to accept, especially as no genetic evidence has so far been established by controlled breeding experiments or from the pedigrees of affected flocks.

CONCLUSIONS

A series of experiments concerned with the agent causing scrapie disease of sheep are described.

The agent was transmitted from the brain, spleen, lymph gland and cerebrospinal fluid of scrapie-affected sheep.

The agent was not recovered from the brain of normal sheep.

The agent was recovered from natural cases of the disease but not from offspring of affected sheep.

The agent was not transmitted by ingestion.

The agent was recovered from sheep following a series of blind passages.

Some of the characteristics of the agent are described.

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AN AID TO THE DIAGNOSIS, PROGNOSIS AND TREATMENT OF SOME DISEASES IN THE PIG AND OTHER SPECIES

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INTRODUCTION

A method for the rapid examination of blood samples by measurements of specific gravity was described, and some preliminary results on its use in cattle were reported by Edwards (1956). The object of this paper is to describe its usefulness on a more extensive scale in the pig, and to elaborate on some potential applications hitherto apparently unrecognised. It is realised that it may be of greater value in canine and feline practice than in large animals. It is suggested, however, that in the latter field it has a definite place in diagnosis.

METHODS

Samples of oxalated whole blood and serum were taken from 46 ailing pigs, submitted by veterinary surgeons for autopsy over a period of eight months. Venepuncture of the anterior vena cava was carried out by the standard method during the course of euthanasia using pentobarbitone sodium. A dose calculated to create deep anaesthesia was injected, blood samples were then taken and a further injection at the same site completed euthanasia. Three blood samples from cattle with chronic undiagnosed conditions and one from a lamb were also examined.

The samples were kept overnight in the refrigerator and then tests were carried out in the following order:— 0·05 ml. amounts of mixed whole blood were dispensed into 5 ml. of 0·1 N NaOH, and three hours later the haemoglobin value was measured using an electrometric modification of the alkaline-haematin-method (Jones and Bennett, 1957). While the specific gravities of the whole blood and the serum were being estimated, the packed cell volume was being determined in Wintrobe tubes spinning at approximately 3,000 r.p.m. for 45 minutes. The red and white cell layers were measured. The specific gravity of the supernatant plasma was then determined; the approximate difference in specific gravity between the serum and the plasma was calculated by subtraction and its significance assessed together with the extent of the white cell layer.

RESULTS

Piglet Anaemia

Table 1 shows the results obtained with pigs suffering from piglet anaemia, haemolytic disease (No. 52) and other conditions. Using the formula designed for man by Phillips, Van Slyke, Hamilton, Dole, Emerson, Archibald and Stanley (1950), the haemoglobin value was calculated from values for the specific

TABLE 1

DIAGNOSIS OF PIGLET ANAEMIA

No.	*Age*	*Wt.*	*Diagnosis*	*S.G. whole blood*	*S.G. serum*	*P.C.V.*	*Haemoglobin grams/100 ml.*		
							electrometric	*by S.G.*	*corrected*
536 (3)	7 wks.	21½ lb.	Scouring, anaemia, rickets, subvitaminosis, B.	1·0385	1·026	20·5	5·5	5·83	4·49
16 (2)	8 wks.	14 lb.	Anaemia and chronic colitis	1·038	1·0245	21·0	5·4	5·98	4·74
191	6 wks.	10 lb.	Severe nutritional anaemia	1·039	1·032	14·0	4·7	4·36	2·97
536 (1)	8 wks.	21 lb.	Severe septic wound, subclinical anaemia (and as in 536 (3))	1·042	1·0275	27·0	6·4	6·74	4·93
16 (1)	8 wks.	12 lb.	Subclinical anaemia and chronic colitis	1·0365	1·022	23·0	6·1	6·23	—
47 (1)	4 wks.	14 lb.	Ectoparasitism, pig pox, subclinical anaemia	1·0435	1·029	30·0	7·7	6·89	5·64
47 (4)	7 wks.	13 lb.	Ectoparasitism, pig pox, V.P.P., inoculation sepsis, and subclinical anaemia	1·038	1·0235	21·0	5·9	5·37	5·54
52	3 days	3½ lb.	Iso-immunisation (Gloucester Old Spot dam)	1·026	1·0215	5·0	1·4	1·82	—
140	8 wks.	20 lb.	Non-nutritional anaemia, endocarditis and septic embolism	1·034	1·0215	16·5	4·9	5·66	—

gravity of whole blood and serum. The difference between the electrometric and calculated values was less than 7 per cent in 29 out of 43 cases fully examined, and less than 13 per cent in 38 of them. Only one sample produced a discrepancy, expressed as a percentage of the electrometric value, of more than 19 per cent. In this case (Pig 52) the haemoglobin level was exceptionally low, the difference between the specific gravities of serum and whole blood being only 0·0045, and therefore probably beyond the limit of sensitivity of the method for the calculation of haemoglobin, where an interval of 0·001 is used. As other methods available to the practitioner are also subject to technical and human error, this seems to be satisfactory for practical purposes. With reference to Pig 52, bearing in mind its clinical condition on examination, the haemoglobin value obtained must be very close to the minimum compatible with life in this species.

Using the following formula the electrometric haemoglobin values were converted into true haemoglobin values using the S.G. of the serum as a basis for calculating the degree of dehydration. Where V ml. of blood are dehydrated to a volume of 100 ml., C. being packed-cell-volume, the true concentration of haemoglobin (Ht) will be:—

$$\text{Haemoglobin found (Hf)} \times \frac{100}{V}$$

$$\text{Now } V = C + V - C$$

$$\text{and } C \equiv 3\,Hf$$

$$\text{but } V - C = \frac{\text{serum protein found (A)}}{\text{normal serum protein (N)}} \times 100 - C.$$

$$\therefore Ht = \frac{Hf \times 100}{3Hf + \left[(100 - 3Hf) \times \frac{A}{N}\right]}$$

According to Phillips *et al.* (1950) the S.G. of serum is convertible into serum protein concentration. Serum specific gravity values in apparently normal pigs, converted into serum protein values using their formula, agreed closely with the normal values quoted by the workers below. Taking 5·38 g. of serum protein/100 ml. of serum as normal for unweaned pigs (McCance and Widdowson, 1957; Rutquist, 1958) and 6·455g/100 ml. of serum as normal for weaned pigs (Garner, Crawley and Goddard, 1957), the ratio of the relevant one of these two values to the value obtained for the serum of a given pig will provide a measure of the degree of dehydration of the plasma. Table 2 gives direct conversions from S.G. serum to percentage water loss from the plasma. The latter was calculated as follows:—

$$\text{Plasma volume after dehydration} = \frac{\text{normal serum protein (N)}}{\text{serum protein found (A)}} \times \text{normal plasma volume (X)}$$

$$\therefore \text{Loss of plasma volume} = X - X\frac{N}{A}$$

$$\therefore \% \text{ water loss} = \frac{X - X\frac{N}{A}}{X} \times 100$$

$$= \left(1 - \frac{N}{A}\right) \times 100$$

TABLE 2

THE CONVERSION OF S.G. SERUM INTO SERUM PROTEIN AND DEGREE OF DEHYDRATION

S.G. of serum	*Equivalent protein (g/100ml.)*	*Potential approximate degree of dehydration*	
		Unweaned	*Weaned*
1·017	3·63		
1·0175	3·81		
1·018	3·98		
1·0185	4·15		
1·019	4·33		
1·0195	4·50		
1·020	4·67		
1·0205	4·85		
1·021	5·02		
1·0215	5·20		
1·022	5·38		
1·0225	5·56		
1·023	5·74	6%	
1·0235	5·92	9%	
1·024	6·10	12%	
1·0245	6·28	14·5%	
1·025	6·46	17%	
1·0255	6·64	19%	
1·026	6·82	21%	5%
1·0265	7·00	23%	7·5%
1·027	7·18	25%	10%
1·0275	7·36	27%	12%
1·028	7·54	29%	14%
1·0285	7·72	30·5%	16%
1·029	7·91	32%	18%
1·0295	8·09	33·5%	20%
1·030	8·27	35%	22%
1·0305	8·47	36·5%	
1·031	8·66	38%	
1·0315	8·85	39·5%	
1·032	9·04	41%	

Dehydration

Conditions in which dehydration was the principal finding on examination of the blood are grouped in Table 3.

Inflammatory Conditions

These are grouped in Tables 4 and 5. Particular note should be given to the white cell component of the packed cell volume and the

TABLE 3

CONDITIONS WITH DEHYDRATION AS THE PRINCIPAL HAEMAL FINDING

No.	*Age*	*Wt.*	*Diagnosis*	*S.G. whole blood*	*S.G. serum*	*P.C.V.*	*Haemoglobin grams/100 ml.*			*Potential plasma water loss*
							electrometric	*by S.G.*	*corrected*	
433	2 days	—	Starvation	1·047	1·026	32	9·8	9·94	8·48	21%
996	4 days	—	Scouring	—	1·026	—	11·4	—	9·62	21%
531	1 days	3 lbs.	Atresia ani.	1·0535	1·028	41	12·1	12·48	9·63	29%
253	2 days	3 lbs.	Atresia ani.	1·0495	1·024	38	11·8	11·94	10·86	12%
519	3 mths.	17 lbs.	Scouring syndrome	1·0525	1·0275	39·5	10·8	12·1	9·87	12%
525	14 wks.	40 lbs.	Focal necrotic colitis	1·0565	1·0285	41	13·3	13·83	11·91	16%
2	4 mths.	50 lbs.	Chronic arthritis (Erysipelothrix and Haemophilus isolated)	1·0535	1·030	35	10·6	11·99	7·9	22%
97	10 wks.	45 lbs.	Confirmed swine fever (symptoms included nystagmus)	1·0575	1·029	45	13·9	14·19	12·28	18%
47 (3)	7 wks.	15 lbs.	Ectoparasitism, pig pox, V.P.P., lingual necrosis and inoculation abscess	1·0435	1·0245	35	9·5	8·61	8·48	14·5%
260	3 wks.	4 lbs.	Exudative dermatitis	1·052	1·032	27·5	9·5	10·53	6·35	41%
276	6 wks.	13½ lbs.	Suspected Talfan disease	1·054	1·0255	47	14·4	14·1	12·74	19%

TABLE 4

INFLAMMATORY CONDITIONS IN PIGS

No.	*Age*	*Wt. lbs.*	*Diagnosis*	*S.G. whole blood*	*S.G. serum*	*P.C.V.*		*S.G. plasma*	*Haemoglobin electrometric*	*S.P.D.*	
						R.B.C's.	*W.B.C's.*			*S.G. —0·0004*	*Equivalent protein grams/100ml.*
47 (2)	4 wks.	13	Ectoparasitism, pig pox, large submaxillary abscess	1·048	1·0265	34	1·5	1·029	11·0	0·0021	0·76
47 (4)	7 wks.	13	Ectoparasitism, pig pox, V.P.P., inoculation sepsis and subclinical anaemia.	1·038	1·0235	21	—	1·0275	5·9	0·0036	1·30
143	5 mths.	50	Inoculation sepsis and necrotic enteritis	1·047	1·0255	34·5	1·2	1·028	10·2	0·0021	0·76
144	3½ mths.	20	Pig pox, umbilical abscess and necrotic enteritis	1·043	1·0225	32	1·2	1·0255	9·1	0·0026	0·94
137	9 wks.	18	Posterior paralysis due to an encephalomyelitis	1·045	1·0225	32·5	2·0	1·0265	10·2	0·0036	1·30
139	8 wks.	14	Septic embolism	1·0425	1·021	30	2·0	1·023	9·2	0·0016	0·58
65	10 wks.	24	Chronic colitis	1·0575	1·024	51	2·0	1·027	15·1	0·0026	0·94
106	11 wks.	22½	Colitis	1·043	1·0215	32	1·5	1·0235	9·9	0·0016	0·58
316	3½ mths.	48	Chronic colitis	1·047	1·0235	34	1·5	1·026	9·8	0·0021	0·76
317	3½ mths.	28	Chronic colitis	1·0425	1·0225	24·5	1·0	1·025	9·0	0·0021	0·76

TABLE 5

INFLAMMATORY CONDITIONS IN OTHER SPECIES

No.	*Age*	*Wt. lbs.*	*Diagnosis*	*S.G. whole blood*	*S.G. serum*	*P.C.V.*		*S.G. plasma*	*Haemoglobin electrometric*	*S.P.D.*	
						R.B.C's.	*W.B.C's.*			*S.G. —0·0004*	*Equivalent protein grams/100ml.*
B 75	—	Cow	Bovine malignant catarrh with Pseudomonad septicaemia	1·0525	1·0275	40	0·7	1·030	11·8	0·0021	0·77
FS 98	—	Cow	Negative for Johnes Disease. (Total white cells 13,300 and shift to the left)	1·052	1·030	32	1·5	1·033	10·6	0·0026	1·00
B 166	—	Cow	—	1·0445	1·0260	24	0·5	1·0295	8·7	0·0031	1·13
S 207	2 mths.	Lamb	Pneumonia (*Pasteurella haemolytica* isolated)	1·0555	1·0295	37	1·0	1·033	13·1	0·0031	1·18

TABLE 6

NEONATAL NUTRITIONAL OEDEMA IN PIGLETS

No.	*Age*	*Wt. lbs.*	*Diagnosis*	*S.G. whole blood*	*S.G. serum*	*P.C.V.*	*Haemoglobin*		*Equivalent serum protein grams/100ml.*
							electrometric	*by S.G.*	
459	24 hrs.	—	Congenital mandibular defect	1·045	1·017	42·5	13·3	11·81	3·63
209	24 hrs.	3	Congenital spinal defect (trace of colostrum in stomach)	1·0425	1·0165	39	12·1	11·04	3·47
7	24 hrs.	—	Empty stomach; very dehydrated	1·0555	1·0195	55	16·6	16·31	4·50

TABLE 7

CONVERSION OF S.G. VALUES INTO CORRECTED HAEMOGLOBIN IN PIGS UNDER 8 WEEKS OLD

Specific Gravity of Serum

Specific Gravity of Whole Blood	1·021	0215	022	0225	023	0235	024	0245	025	0255	026	0265	027	0275	028	0285	029	0295	030	0305	031	0315
1·035	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4	2·1	1·9	1·7	1·5	1·3
1·0355	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4	2·1	1·9	1·7	1·5
1·036	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4	2·1	1·9	1·7
1·0365	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4	2·1	1·9
1·037	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4	2·1
1·0375	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6	2·4
1·038	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8	2·6
1·0385	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0	2·8
1·039	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2	3·0
1·0395	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4	3·2
1·040	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7	3·4
1·0405	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9	3·7
1·041	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1	3·9
1·0415	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3	4·1
1·042	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5	4·3
1·0425	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7	4·5
1·043	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0	4·7
1·0435	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2	5·0
1·044	9·8	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4	5·2
1·0445	10·0	9·8	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6	5·4
1·045	10·2	10·0	9·8	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8	5·6
1·0455	10·5	10·2	10·0	9·8	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1	5·8
1·046	10·7	10·5	10·2	10·0	9·8	9·6	9·4	9·1	8·9	8·7	8·5	8·3	8·0	7·8	7·6	7·4	7·2	6·9	6·7	6·5	6·3	6·1

serum plasma difference. In three cases blood samples from adult cattle have been examined and one sample from a lamb. The S.P.D. gives useful results, whereas the E.S.R. in cattle is valueless, due to the physical properties of the blood (Rankin, 1955).

Neonatal Nutritional Oedema

Death from starvation is recognised as a very common condition and is presumed to be due mainly to glucose deficiency. Low serum protein values, normally remedied by the ingestion of colostrum, have been described in normal pigs by McCance and Widdowson (1957) and Rutquist (1958). It is possible that "jelly" pigs are an extreme form of this normal early neonatal condition of gross protein deficiency. Table 6 illustrates the very low serum S.G. found in pigs unable to ingest colostrum due to congenital abnormalities and other conditions, and serves to confirm the findings of the workers quoted.

DISCUSSION

In devising prophylactic measures for piglet anaemia, each case must be treated on its merits, due regard being given to the system of management and the actual disease picture in each herd (Bennett and Edwards, 1958). With this object in view it is often valuable to assess the haemoglobin status of the pigs involved. It is hoped that the specific gravity method may be practicable where conventional methods are too time consuming. By the use of Table 7 true haemoglobin may be read off from values for the S.G. of serum and whole blood without any calculations. Outbreaks of clinical piglet anaemia are frequently complicated by scouring and some degree of dehydration, giving falsely high haemoglobin values. Hence the table is arranged to make allowance for this error in determining haemoglobin concentrations, and it is hoped that the differential diagnosis of anaemia from other conditions causing scouring will be made easier.

The extent of the serum-plasma difference was a rather unexpected finding. Although it must represent the level of fibrinogen present in the blood at clotting, its value appears to be normally quite high as compared with man. The normal difference between the specific gravities of serum and oxalated plasma has been found to be of the order of 0·0015. To allow for the error due to oxalate added to the plasma, 0·0004 should be subtracted from this value, leaving an S.P.D. of 0·0011 which is equivalent to 0·39g. of protein/100 ml. No figures seem to be available on the normal fibrinogen level in pigs, but considering that the value accepted for man is 0·25 to 0·28g/100 ml. the level is of the same order, if rather higher. It is definitely raised in simple septic conditions and in some chronic conditions involving the large intestine. This fits in with the view held by medical pathologists that raised plasma fibrinogen levels, as indicated by an increase in erythrocyte sedimentation rate are a

sign of inflammation or tissue destruction (Wright, 1953). It is unfortunate, but implicit in the methods used, that the fibrinogen level is neglected in paper electrophoresis on serum samples. Specific gravity methods might possibly be used to remedy this deficiency.

Although fluid replacement therapy in the pig is probably impracticable under present conditions, Table 2 is appended to show how the degree of dehydration may be calculated readily from the specific gravity of serum. Walker (1956) considers that a formula based on haemoglobin or haematocrit values may be used in the assessment of dosage for the easement of haemoconcentration by fluid replacement therapy. Due to the normal and pathological variation in these components of the blood irrespective of the condition under treatment, a further unrelated test might be useful as a check. Furthermore in cats, in an advanced state of dehydration, it is probably easier to obtain small samples of serum than samples of unclotted and representative whole blood.

CONCLUSIONS

Specific gravity methods are shown to be of diagnostic value in piglet anaemia, and they appear to be of value in the assessment of dosage for fluid replacement therapy. They are simple to execute and require no specialised or expensive apparatus. The normal fibrinogen level in the pig is apparently higher than that recorded for man. The serum plasma difference is raised in septic conditions and in chronic conditions of the large intestine in the pig. As a more direct approach to fibrinogen level, it appears to be a potentially useful alternative to the E.S.R., particularly in cattle. Recent experimental findings demonstrating very low serum protein levels in neonatal pigs may be confirmed in the field by using estimations of serum specific gravity.

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THE BLOOD GROUPS OF THE PIG

VII. The Distribution of Twelve Red Cell Antigens in Seven Breeds

By

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INTRODUCTION

In a communication to the VIIth International Congress of Animal Husbandry, Irwin (1956) stated, 'A fertile field for research in all species of domestic animals, following the recognition of antigenic characters within any species, is a comparison of the frequencies of the various genes with antigenic effects in the different breeds. From such studies would come a relatively precise measure of the evolution of the various breeds'. This paper concerns the frequency of blood-group antigens in a number of pigs of seven pure breeds.

Immune reagents were available for defining the A and eleven other red cell antigens (Joysey, Goodwin and Coombs, 1959). Using these, a start has been made in characterizing different breeds of pigs by their blood-group patterns, and it was hoped that this might throw light on the observation of Goodwin and Saison (1956) that there is a breed difference in the iso-antibody response evoked by crystal violet swine fever vaccine. In turn, as most litters affected with haemolytic disease of the new-born are from certain breed crosses, the frequency of antigens in these breeds might suggest which antigens cause the disease. There was the further possibility that by using a particular breed of pig for vaccine production the incidence of haemolytic disease might be reduced.

MATERIALS AND METHODS

Selection of pigs. The breeds sampled were Essex, Wessex, Large White, Landrace, Large Black, Tamworth and Gloucester Old Spot. All the pigs were purebred; that is, eligible for the Herd Books if not actually entered. The original aim was to collect at least fifty samples from each breed but this was not fulfilled with the Tamworth breed or, more notably, with the Gloucester Old Spots.

The selection within the breeds was designed to obtain as representative a picture of each breed as possible, in order that local fluctuations of gene frequency due to inbreeding might be swamped. To this end, rarely were more than twelve pigs sampled in any one herd and the farms visited, although mainly in the Eastern Counties and the Midlands, were well scattered over this area and beyond. The intention was not to bleed litter-mates, or both a parent and its offspring, but this latter relationship was sometimes included. Thus, although when visiting each new farm the animals previously sampled were kept in mind, it was occasionally found later that parents and offspring had, in fact, been

bled on separate farms. Whenever possible, pigs were not bled if they had a common parent but it was difficult to avoid this relationship in several herds. However, if there were a choice of relationships of this type the pigs sampled were those where the two parents other than the shared one were least related. Table 1 shows the number of pigs and herds sampled and the number of samples taken from each herd in the seven breeds.

TABLE 1

COMPOSITION OF BREED SAMPLES

Total number of pigs and herds sampled in each breed, together with the number of pigs sampled in each herd.

Breed	*Total pigs tested*	*Total herds tested*	*Number of samples from each herd*
Essex	64	7	9, 6, 10, 9, 10, 10, 10.
Wessex	57	8	11, 10, 8, 2, 4, 10, 10, 2.
Large White	64	9	6, 4, 5, 3, 10, 10, 6, 10, 10.
Landrace	71	8	4, 2, 10, 10, 10, 12, 11, 12.
Large Black	66	7	14, 8, 11, 4, 12, 5, 12.
Tamworth	43	8	10, 3, 7, 6, 9, 2, 5, 1.
Gloucester Old Spot	14	2	8, 6.

The degree of relationship existing between all the samples within each breed cannot be readily assessed, but certain broad impressions emerge. Excluding the few samples from the Gloucester Old Spots, the Tamworth samples seemed the most closely related, followed by the Large Blacks. The Essex and Wessex samples were roughly comparable, the more frequent parent-offspring sampling in the Wessex breed being compensated for by the number of pigs with a common parent in the Essex breed. The Landrace and Large White pigs were, superficially, much less closely related, particularly the Large Whites, where the numerically large size of the breed gave a far greater freedom of choice before sampling. This variation in the relationship of the sampled pigs is a reflection of the relative size of the breeds; the Tamworth breed, for example, being very small in numbers, while the Large Blacks are more common than Tamworths but considerably less popular than the Essex/Wessex breeds. As a corollary to this, however, it should be noted that the method of selecting the samples was more applicable to the larger breeds, because the closest relationships were deliberately avoided and these figure more prominently when the breed numbers are small.

Serological tests. The preparation of the reagents, and the testing

procedures were as previously described (Joysey *et al.*, 1959). The anti-globulin sensitization test was used throughout. Where the result of a test was equivocal the test was repeated, and if the result was still not clear-cut it was entered as unknown(?). Table 2 shows that anti-7 gave equivocal results more frequently than the other sera and although this reagent appears to be of single specificity it is not entirely satisfactory (Joysey *et al.*, 1959).

TABLE 2

DISTRIBUTION OF ANTIGENS

Results of tests with twelve antisera against the red cells from seven breeds of pigs.

Breed		*Antisera to Antigens*											
		A	1	2	3	4	5	6	7	8	9	10	11
Essex	+	31	14	18	52	5	13	38	2	21	62	16	26
	—	32	50	46	12	59	51	26	58	43	2	33	21
	?	1							4				2
Wessex	+	45	18	26	54	4	5	29	5	30	55	15	14
	—	10	39	30	3	53	52	28	47	27	2	11	12
	?	2		1					5				
Large White	+	22	29	15	60	5	17	62	7	0	64	9	22
	—	42	35	49	4	57	47	2	52	64	0	45	32
	?					2			5				
Landrace	+	14	54	8	72	10	23	69	13	6	72	8	3
	—	58	17	63	0	58	48	2	56	66	0	63	68
	?		1	1		4	1	1	3			1	1
Large Black	+	38	38	32	45	26	41	57	21	21	65	59	30
	—	28	27	34	21	35	25	9	42	45	1	6	36
	?		1			5			3			1	
Tamworth	+	6	18	26	28	0	0	38	6	41	21	37	30
	—	37	25	17	15	43	43	5	29	2	22	6	11
	?								8				2
Gloucester Old Spot	+	9	12	6	10	0	0	10	5	2	14	3	11
	—	5	2	8	4	14	14	4	9	12	0	11	3
	?												

Fig. 1

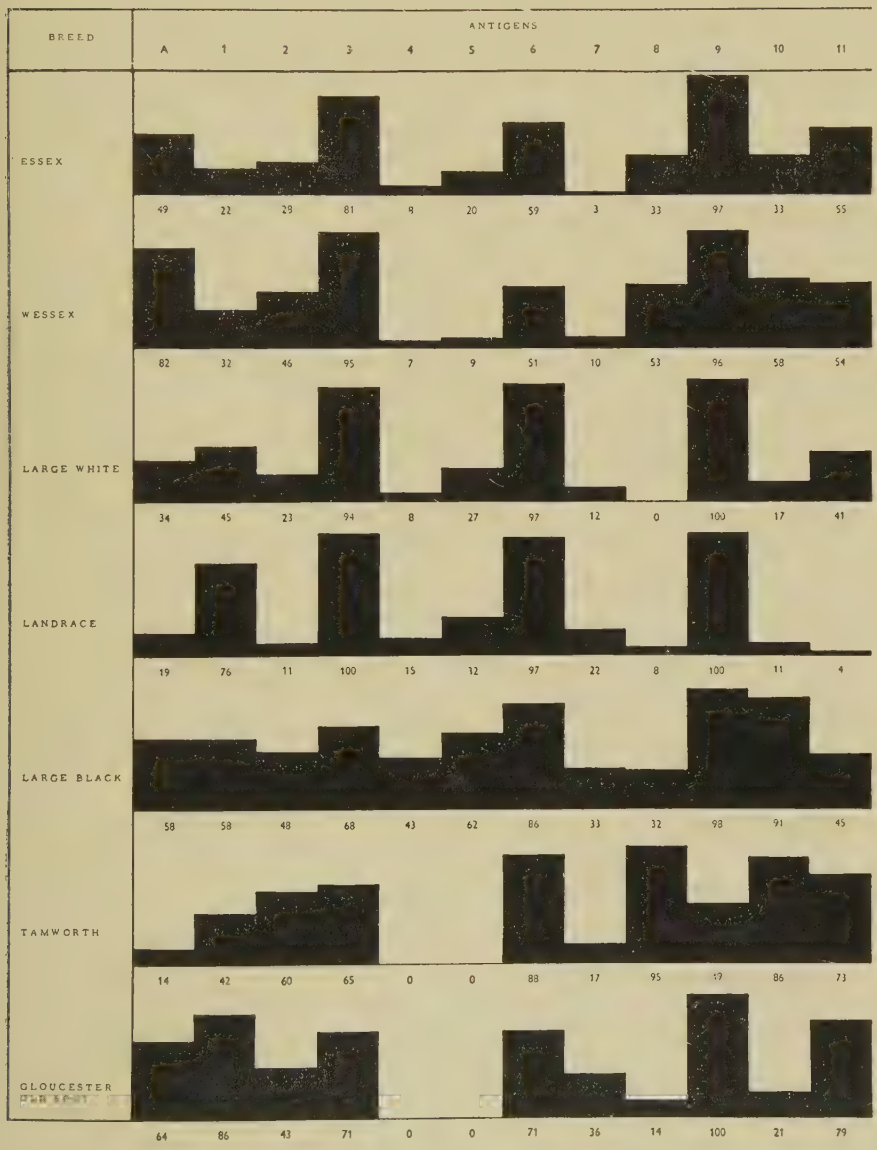

FIG. 1. THE PERCENTAGE FREQUENCY OF TWELVE RED CELL ANTIGENS IN SEVEN BREEDS OF PIGS.

NOTE. THE FIGURES BELOW EACH HISTOGRAM DENOTE THE PERCENTAGE OF ANIMALS POSSESSING THE ANTIGEN CONCERNED.

TABLE 3

FREQUENCY RANGES OF ANTIGENS

The frequency ranges of the twelve antigens placed in increasing order.

Antigen	*Lowest frequency* (%)	*Frequency range*
7	3	33
3	65	35
4	0	43
6	51	46
2	11	49
9	49	51
5	0	62
1	22	64
A	14	68
11	4	75
10	11	80
8	0	95

RESULTS

The results of the tests using twelve sera on the red cells from seven breeds of pigs are shown in Table 2. The same results are also expressed as percentages in a series of histograms (Fig. 1), all equivocal tests being omitted. As the survey was started before

reagents anti-10 and anti-11 were prepared, a number of the samples from the Essex, Wessex and Large White breeds were not tested against these two reagents. It should be noted that Table 2 and Fig. 1 refer to phenotype frequencies; it is not possible at this stage to determine the gene frequencies as the inheritance of the antigens is unknown. One of the main points of interest is the range of frequency of the antigens in the different breeds and these are listed in increasing order in Table 3.

DISCUSSION

Fig. 1 shows that antigen 5 regularly occurs at a higher frequency than antigen 4, and it is noteworthy that antigen 4 does not occur in the absence of antigen 5. This could be an effect similar to that envisaged by Ferguson (1941) with the C and E antigens in cattle, where only two combinations were possible, i.e. C and E together or C alone. Alternatively, it is known that our anti-5 serum is contaminated with anti-4, hence it is possible that some of the animals classed as 4 + 5 +, may be 4 + 5 —. Repeated efforts to find a 4 + 5 — animal, however, in order to produce a pure anti-5, have been unsuccessful, and hence the former explanation seems the more likely.

Despite the similar appearance of the Essex and Wessex breeds, they do not have the same antigen frequencies. The frequency of antigens A, 2, 8 and 10 in Wessex pigs is considerably higher than in Essex pigs (A: 82 per cent and 49 per cent, 2: 46 per cent and 28 per cent, 8: 53 per cent and 33 per cent, and 10: 58 per cent and 33 per cent, respectively). The lowest frequency of antigen 6 occurred in these two breeds (Essex 59 per cent, Wessex 51 per cent).

Large White pigs were outstanding in that no pig possessed antigen 8, and in this breed antigens 3, 6 and 9 all have a very high frequency (94 per cent, 97 per cent and 100 per cent, respectively). The blood groups of the Landrace pigs broadly resemble those of the Large Whites, in that they also have a low frequency of antigen 8 (8 per cent) and very high frequencies of antigens 3, 6 and 9 (100 per cent, 97 per cent and 100 per cent).

Of the Large Black pigs, 43 per cent possessed antigen 4. This antigen was rare or absent in the other breeds tested, its next highest frequency being 15 per cent in the Landrace pigs.

Both Tamworth and Gloucester Old Spot pigs lacked entirely antigens 4 and 5. The Tamworth pigs were also notable in possessing the highest frequency of antigen 8 recorded (95 per cent) and the lowest frequency of antigen 9 (49 per cent). The latter figure does not seem to be a very low frequency until it is compared with the second lowest frequency of 96 per cent in the Wessex breed.

All the antibodies, other than anti-A, used to detect the antigens in this survey were derived from sows that had affected their litters

with haemolytic disease, but some antibodies are more likely to have caused the disease than others. For example, anti-3, -6 or -8 often occur in haemolytic sera, and they tend to be of high titre (greater than 128). Furthermore, incompatability for the corresponding antigens can often be demonstrated between the sow and boar. Anti-1, -2, -4, -5 and -7 tend to be of lower titre, while anti-10 and anti-11 were the last antibodies to be isolated and can be presumed to be less common; all these, therefore, are not so likely to be the causative antibodies. Only one example of anti-9 has been found; this was of high titre and probably caused the disease. As by far the largest number of cases of haemolytic disease occur in the Essex or Wessex ♀ x Large White ♂ cross, the disease will now be considered in relation to the antigen frequencies of these three breeds.

Antigen 8 is heavily implicated in some cases of haemolytic disease (Joysey *et al.*, 1959) but it could not be the operative antigen in the Essex/Wessex x Large White matings as no Large White pig has been found to possess antigen 8. One might speculate, however, that when an allele to the gene responsible for the production of antigen 8 is found, its antigen may prove to be one of the main causes of haemolytic disease in these particular breed crosses. Of the other antigens, 9 has been implicated in one case of haemolytic disease but as its incidence is high in all the breeds studied, other than the Tamworth, it could rarely be the responsible antigen. Antigen 3 is a more likely cause of incompatability and the corresponding antibody has been found at high titre in several sows that had had clinically affected litters (Joysey *et al.*, 1959). Nevertheless, the high incidence of the antigen (Essex 81 per cent, Wessex 95 per cent and Large White 94 per cent) suggests that it is not the main answer to this problem.

In contrast to the antigens just discussed, the frequency of antigen 6 in the three breeds suggests that this may be a major cause of the disease. This antigen is present in 97 per cent of Large White pigs, while it occurs in only 59 per cent and 51 per cent of Essex and Wessex pigs, respectively. Nearly 50 per cent of the Essex or Wessex x Large White matings, therefore, will be incompatible for antigen 6. Were it not already known that other factors limit the number of clinical cases of haemolytic disease (Goodwin and Saison, 1957), such a distribution of antigen 6 could be expected to result in heavy neonatal mortality.

Haemolytic disease occurs in purebred Essex or Wessex piglets but is strikingly absent among purebred Large Whites. This observation led Goodwin and Saison (1956) to compare the incidence of iso-antibodies in these breeds. Finding that iso-antibodies to the red cells of Large White boars were uncommon among sows of the same breed, these authors postulated that Large Whites possessed almost universally one or more antigens not generally present in Essex or Wessex pigs. They further suggested that the

Large White breed was very homogeneous in its blood-group structure, and this is borne out by the present work. With regard to antigens 3, 6, 8 and 9, the Large White breed is remarkably homogeneous (3: 94 per cent, 6: 97 per cent, 8: 0 per cent, and 9: 100 per cent) and intra-breed matings will rarely be incompatible in this respect. Nevertheless, a number of Large White sows do produce iso-antibodies to the red cells of Large White boars (Goodwin and Saison, 1956) and it is not entirely clear why, when the Large White breed is the most common breed in the country, no clinical case of haemolytic disease has been recorded among the litters of these sows. This anomaly is now being investigated further.

There is no evidence to suggest that haemolytic disease in the pig is other than an artificial condition induced by the injection of crystal violet swine fever vaccine. But only the litters from the black breeds appear to be at obvious risk and the major part of these are from Essex and Wessex sows. If the latter could be injected with compatible blood, therefore, the disease would be economically insignificant. The vaccine is made from the bulked blood of about fifty pigs, but even with the small number of blood groups known at present there is no single breed that could supply sufficiently compatible blood. Any manoeuvre more complicated than the selection of one breed for vaccine production could not be economically justified when, compared with haemolytic disease, swine fever is by far the greater evil.

CONCLUSIONS

The distribution of the A antigen and eleven other red cell antigens has been studied in seven breeds of pigs. The breeds sampled were Large White, Landrace, Wessex, Essex, Large Black, Tamworth and Gloucester Old Spot.

The blood-group patterns of these various breeds are discussed in relation to those antigens most probably concerned in haemolytic disease of the new-born.

ACKNOWLEDGMENTS

We are grateful to Mr. P. J. D. V. Brett and Mr. R. H. Matthews for technical assistance. This work could not have been undertaken without the willing co-operation of many breeders of pedigree pigs: to all these farmers we express our thanks. We wish to acknowledge also the continuing financial support of the Agricultural Research Council.

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EXPERIMENTAL PRODUCTION OF SCRAPIE IN GOATS

By

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INTRODUCTION

The experimental transmission of scrapie to sheep was first achieved by Cuillé and Chelle in France in 1936; it was repeated on an extensive scale by Gordon, Brownlee and Wilson (1940), and has since been confirmed by other workers (see Gordon, 1957). Cuillé and Chelle (1939) also succeeded in transmitting the disease to two goats, the only two inoculated, by intraocular injection of a suspension of lumbar cord from an advanced natural case of scrapie. One of the goats was a two-year-old female and the other a six-month-old castrated male, and symptoms of the disease did not appear until 26 months after inoculation. The authors noted that the symptoms shown by the goats were those of trembling and locomotor disturbance, and that the dorso-lumbar pruritis that is so characteristic of many cases of scrapie in sheep was "totally absent." Chelle (1942) reported a case of scrapie in a goat that had been in close contact with affected sheep, and in this case lumbar pruritis was present in addition to locomotor disturbance.

The experimental production of scrapie in goats following intracerebral inoculation of brain suspension from a scrapie-affected sheep was noted by Pattison (1957). Gordon and Pattison (1957) drew attention to the apparent high degree of susceptibility of the goat—as compared with the sheep—to intracerebral inoculation with scrapie brain suspension or cerebro-spinal fluid. Both of these brief reports were in the nature of preliminary communications, and this present paper enlarges them by describing scrapie as produced experimentally in goats following intracerebral inoculation of brain suspension or cerebro-spinal fluid from scrapie-affected animals.

MATERIALS AND METHODS

Scrapie material used. The suspension of scrapie sheep brain used to produce the disease in goats, was obtained in 1953 from Dr. D. R. Wilson of Moredun Institute. This "strain" of scrapie had been isolated from naturally-affected South Scotland Cheviot sheep, and had been passaged several times through sheep at Moredun before being sent to Compton. At Compton, one intracerebral passage through South Scotland Cheviot sheep and a second passage by the same route through a Welsh Mountain sheep were made before the first goats were inoculated in 1954.

Inocula. In the various experiments described below, the intracerebral inocula used were either brain suspension or cerebro-spinal fluid; the volume inoculated was 1·0 ml. in every case. The brain inoculum was prepared by grinding pieces of brain-stem, cerebellum and cerebral cortex to a paste in a mortar, with a little sterile sand, and adding

sufficient physiological saline to make a 10 per cent weight/volume suspension. This was centrifuged at about 1,500 r.p.m. for 15 minutes and the supernatant was used as the inoculum. Inoculations were always made within 24 hours of preparation of the inoculum. Cerebro-spinal fluid was obtained by puncture through the cisterna magna under nembutal anaesthesia; up to 10 ml. of fluid were withdrawn at any one operation.

Histology. Tissues for histological examination were fixed in 10 per cent formol-saline and embedded in paraffin wax. Sections were stained with haematoxylin and eosin.

Goats. The goats were from the Compton herd of cross-bred animals, housed indoors in loose-boxes in the isolation compound. Females or castrated males of various ages were used in different experiments, as indicated under "Results."

RESULTS

Experimental Production of the Disease

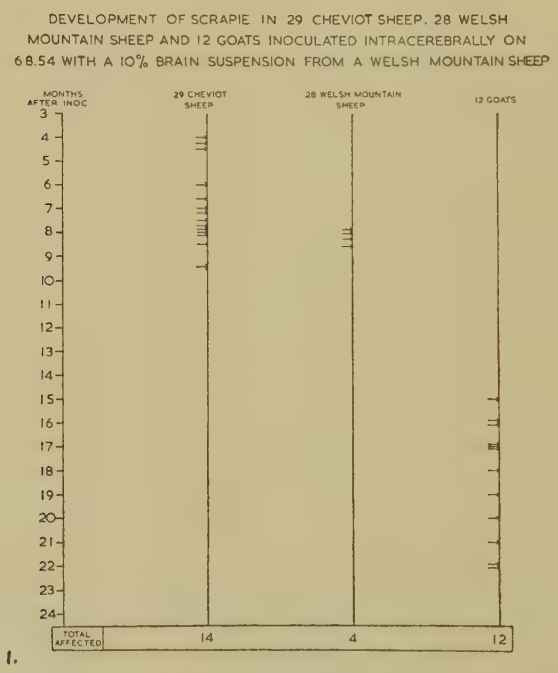
Intracerebral inoculation of goats with brain material from a normal sheep. As controls for the various experiments described below, five female goats and five castrated males all approximately six months old were inoculated intracerebrally with a 10 per cent suspension of brain from a normal Welsh Mountain sheep and were kept under observation for 22 months, during which time they remained normal in every respect. The females were mated twice and produced normal offspring. This 22-month period of observation was equal to the longest scrapie incubation period that occurred in other experiments. At the end of this time these normal goats were destroyed for histological examination of the medulla oblongata.

First experimental production of scrapie in goats. Of 30 Welsh Mountain sheep inoculated intracerebrally with brain emulsion from an experimentally infected South Scotland Cheviot sheep showing clinical symptoms of scrapie, only one developed the disease, eight months after inoculation. Brain suspension from this animal was inoculated intracerebrally into 29 South Scotland Cheviot wethers, 28 Welsh Mountain wethers and 12 castrated male goats aged about one year on the same day from the same flask of material. The results are given in Fig. 1, from which it will be seen that 14 Cheviot sheep developed the disease between four and ten months after inoculation, four Welsh Mountain sheep were affected between eight and nine months after inoculation, and the disease appeared in all the goats between 15 and 22 months after inoculation. The sheep that did not develop the disease were kept under observation for two years.

Second passage of scrapie from goats to goats, and passage from goats back to sheep. Brain suspensions were made from the first two goats to be affected with scrapie (Fig. 1) and goats and sheep were inoculated intracerebrally with this material. Group 1. Brain suspension

from the first goat was used to inoculate ten South Scotland Cheviot wethers, ten castrated male goats about nine months old, and six male goats, later castrated, between one and seven days old. Group 2. Brain suspension from the second goat was used to inoculate ten South Scotland Cheviot wethers and ten female goats about nine months old. A non-inoculated male was put with these females immediately after they were inoculated, and five produced offspring during the experimental period. The results of these inoculations (Groups 1 and 2) are given in Fig. 2, from which it will be seen that eight of the 20 sheep, (four out of ten in each group) developed the disease between four and 11 months after inoculation, and that all 26 goats became affected between seven and 21 months after inoculation.

Fig. 1



Third and fourth passages from goats to goats. A third passage of scrapie through goats was carried out by intracerebral inoculation of six females about six months old with a suspension of the pooled brains from eight of the scrapie-affected animals of the second passage. A non-inoculated male was put with these females immediately after inoculation and five of them kidded during the experimental period. All six animals developed scrapie between seven and 12 months after inoculation (see Fig. 3).

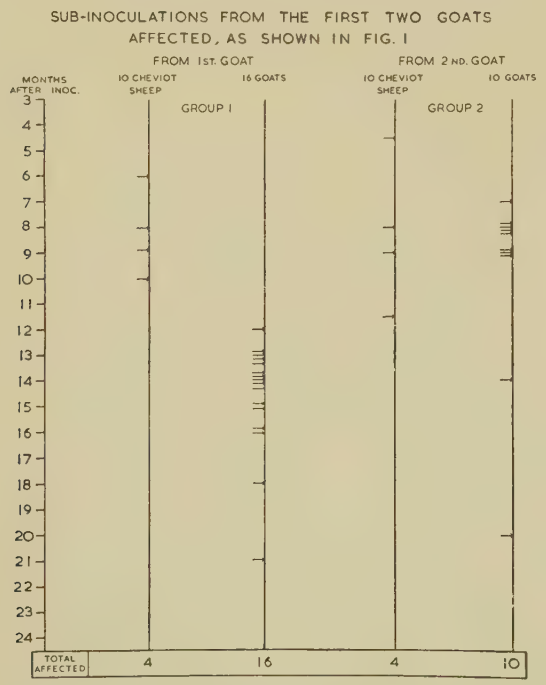
A fourth passage was made by intracerebral inoculation of six

castrated males about four months old with brain suspension from the first third-passage animal to develop the disease (Fig. 3). The first symptoms of scrapie appeared in one animal of this group seven months after inoculation, and five animals were clinically affected by the ninth month (see Fig. 3). Sixteen months after inoculation the sixth animal has developed the disease.

These passages cover the period 1954 to 58, but it is hoped to continue further passages in the future.

Transmission of scrapie from goat to goat by intracerebral inoculation of cerebrospinal fluid. Three four-month-old goats (two females and one castrated male) were inoculated intracerebrally with 1·0 ml. of cerebrospinal fluid obtained from a scrapie-affected goat of the second passage. All animals developed scrapie 10 months later.

Fig. 2



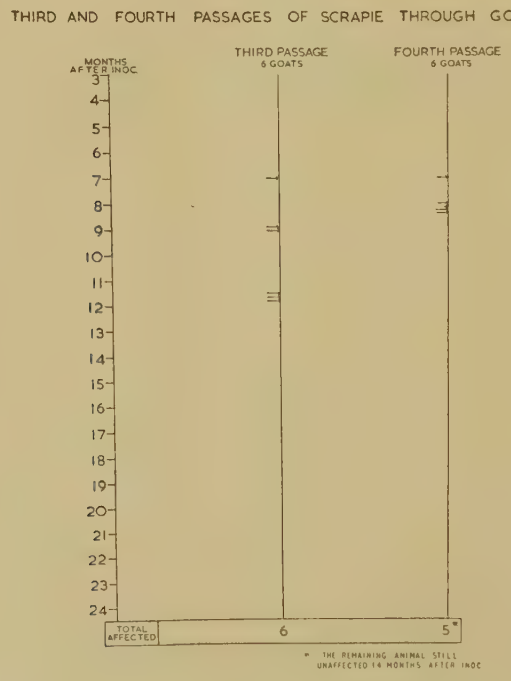
2.

The Clinical Appearance of Goats Affected with Scrapie

Observation of scrapie produced experimentally in goats has shown certain differences between the clinical manifestations of the disease in goats as compared with sheep, thus confirming the finding of Cuillé and Chelle (1936). The characteristic rubbing against fixed objects that is so frequently seen in sheep was unusual in goats. However, several goats showed areas of broken hair produced by

scratching with their horns or hind hooves, and there was a general tendency for the normal "grooming" nibbling and scratching to be increased, suggesting some degree of skin irritation. A very common clinical abnormality in the goat that has not been observed in sheep was cocking of the tail over the back (Figs. 4 and 5); this became more pronounced as the disease progressed, until a stage was reached during which the tail was permanently twisted tightly forwards. The characteristic posture of affected goats—again not seen in sheep—is also shown in Figs. 4 and 5. The hind-legs became drawn under the body so that the hind-quarters appeared raised and the withers appeared dropped, the ears were invariably held forwards, as illustrated, and the anxious expression became permanent.

Fig. 3



Development of the clinical disease in the goat was always a gradual process, so that it was difficult to say exactly when the earliest abnormalities were manifest. On a number of occasions the first sign in a group of animals was a subtle change from the usual friendly, interested temperament to a quietness and a loss of inquisitiveness that is unnatural in healthy goats. Gradually developing restlessness was noted a few days after this quiet period, with an inclination to excessive movement, sudden twitchings of the tail, stamping of the feet, shaking of the head and increasing belligerence

of the animals towards each other. A commonly observed behaviour pattern at this time was for a goat to stop suddenly, lower its head sharply with the ears pricked forward, stay momentarily in this posture as if staring at something on the ground, then to stamp one or more feet and return again to apparent normality. This behaviour is similar to that seen occasionally in cattle and sheep when flies are troublesome.

There was considerable variation among individual goats in the time taken for further clinical abnormalities to become obvious, and two to three months was a usual period to elapse between the earliest symptoms and the end-stages of the disease. The early signs that may be described collectively as irritation gradually gave way to more pronounced signs of disease. There was increasing evidence of hypersensitivity and fear of being handled, manifest in extreme cases by terrified bleating and muscular rigidity when the animal was caught for examination. The tail gradually became more cocked and the posture more abnormal, as described above, and the ears were pricked forward continuously. In some cases there was apparently impaired vision, so that the animal would stare apprehensively at the feet of someone entering the loose-box rather than at the body as a whole, as if aware of an abnormal presence without being able to locate it properly. Gradually hypersensitivity gave way to drowsiness and unsteady gait, and at this stage head tremors were frequently well marked. Characteristically, a sudden noise would startle the animal so that it would jerk its head sharply and marked tremors of the head would continue for a minute or so; then the head would gradually sink, the eyes would half-close, and the animal would sway from side to side, sometimes almost falling before regaining equilibrium (Fig. 6). Not infrequently two goats in this stage of the disease would be observed leaning against each other for support. In many respects the behaviour at this time was similar to that of a drunken man.

The final stage of the disease was collapse, apparently due to failure to maintain equilibrium rather than to paralysis of the limbs. At this time all animals were slaughtered. Appetite appeared to be maintained throughout the disease, and although some animals became thin as the disease progressed, the bodily condition of others at the time of slaughter was good. Once clinical symptoms had appeared, the disease progressed as described, and although, for humanitarian reasons, all animals were slaughtered, there was no indication that there could have been any other than a fatal termination to the disease.

Two cases occurred that were exceptional as to clinical symptoms. Both these animals were inoculated in the second goat passage (Group 2). No early symptoms were noted in these cases, and in both animals locomotor abnormality appeared suddenly, in one case 14 months and in the other 20 months after experimental inoculation (Fig. 2). Both animals went down and had to be des-

troyed approximately ten days after the appearance of clinical abnormality. It may be recorded, without necessarily attaching significance to the fact, that both these animals were in a condition of what may be called physiological abnormality at the time of appearance of locomotor disturbance, in that one was heavily in kid and had dead twins removed by Caesarian section two days before death, and the other showed clinical evidence of hermaphroditism, confirmed post mortem, although at the time of experimental inoculation it was believed to be a female.

It has been mentioned that experimentally-produced scrapie in goats differed clinically from the disease as observed in a majority of sheep, and it should also be recorded therefore, that when sheep were inoculated intracerebrally with brain suspension from one or other of two affected goats (Fig. 2) the disease produced in the sheep was of the characteristic rubbing variety seen in many field cases.

In Table 1 particulars are given of the incubation period in experimentally-produced scrapie, as observed in 52 goats in six experiments. There seemed to be an optimum incubation period within which a majority of animals in that particular experiment became affected. Thus, in the first-passage group this was about 17 months; in one of the second-passage groups it was about 14 months, while in the other second-passage group it was about eight months; in the third-passage group it was about ten months; in the fourth-passage group it was about nine months; and in the cerebro-spinal fluid group it was ten months.

Relative Susceptibility of Sheep and Goats to Experimental Inoculation with Scrapie-infective Material

All workers who have attempted to reproduce scrapie by experimental inoculation of sheep are agreed that the take following intracerebral or subcutaneous injection of scrapie-infective material is usually low and has rarely reached 60 per cent. The take within two years of inoculation recorded by Gordon (1957) in a group of 60 South Scotland Cheviot sheep inoculated subcutaneously with a 10 per cent suspension of scrapie brain, cord and spleen was 13 per cent; in another group of 60 sheep inoculated subcutaneously with the supernatant fluid of a suspension of scrapie brain, cord and and spleen centrifuged at 3,000 r.p.m. it was 18 per cent; and in a group of 30 sheep inoculated intracerebrally with a 20 per cent suspension of scrapie brain and cord it was 43 per cent. By contrast, 52 out of 53 goats inoculated intracerebrally with scrapie brain suspension or scrapie cerebro-spinal fluid have developed the disease; the animal not yet affected has, at the time of writing, been under observation for only 14 months. The incubation period in goats has varied from seven to 22 months, and the distribution of 52 cases according to incubation period is given in Table 1.

TABLE 1

DEVELOPMENT OF CLINICAL SYMPTOMS OF SCRAPIE

52 goats inoculated intracerebrally with brain emulsion or cerebro-spinal fluid from scrapie-affected sheep or goats.

Description of experiment	*No. of animals*	*Incubation period: no. of cases: months after inoculation*														
		7	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*
First passage	12									1	2	3	1	1	1	1	2
Second passage (Group 1)	16						1	4	5	2	2		1			1	
Second passage (Group 2)	10	1	4	3					1						1		
Third passage	6		1	2		1	2										
Fourth passage	5		1	4													
Cerebro-spinal fluid	3				3												

Histological Examination of the Medulla Oblongata of Clinically Healthy and Scrapie-affected Goats

Examination of sections of the medulla oblongata from 45 normal and 22 scrapie-affected goats (Table 2) has indicated that

the positions of the cell nuclei in the medulla of the goat, and the appearance of the cells within the nuclei, are closely similar to those described in the sheep by Pattison and Holman (1943). It seems justifiable, therefore, to use the same terminology for the nuclei in the medulla oblongata of both goat and sheep.

TABLE 2

EXAMINATION OF THE MEDULLA OBLONGATA OF HEALTHY AND SCRAPIE-AFFECTED GOATS FOR VACUOLATED NERVE CELLS

Group of animals	*No. in group*	*No. of sections examined*	*No. of animals in which the average no. of vacuoles per section was:—*				
			0	*<1*	*1–5*	*6–10*	*over 10*
Clinically healthy, non-inoculated goats from the Compton flock.	24	813	16	8	0	0	0
Clinically healthy, non-inoculated goats not from the Compton flock.	11	198	8	3	0	0	0
Clinically healthy goats of the Compton flock slaughtered 22 months after intracerebral inoculation with brain suspension from a normal Welsh Mountain sheep.	10	180	6	4	0	0	0
Advanced experimentally-produced clinical cases of scrapie in goats of the Compton flock.	22	396	0	0	3	4	15

The literature on the occurrence of vacuolated nerve cells in the medulla oblongata of sheep showing clinical evidence of scrapie has been reviewed by Gordon (1957), and the presence of similarly vacuolated cells in experimentally infected goats was recorded by Pattison (1957). Examination of further goat material has revealed vacuolated nerve cells in the medulla oblongata of all goats clinically affected with scrapie. Vacuolated nerve cells are sometimes found in the medulla oblongata of clinically healthy sheep (Holman and Pattison, 1943; Zlotnik and Rennie, 1957), notably in the dorsal motor vagus nucleus (Palmer, 1957), and on rare occasions vacuolated nerve cells have also been found in clinically healthy goats. The finding of vacuolated nerve cells cannot, therefore, be considered pathognomonic of scrapie in either the sheep or the goat, but the large number of vacuolated cells found in advanced clinical cases of scrapie in the goat leaves no doubt that this pathological

change is in some way associated with the disease. Figures for counts of vacuolated cells in (a) clinically healthy, non-inoculated goats from the Compton flock (b) clinically healthy, non-inoculated goats from outside the Compton flock (c) clinically healthy goats inoculated intracerebrally 22 months previously with brain suspension from a normal Welsh Mountain sheep (d) scrapie-affected goats, are given in Table 2. The association between vacuolation of cells and the presence of clinical scrapie is obvious. There was no evidence that previous inoculation with normal brain suspension tended to produce vacuolation of nerve cells, and as all the material was prepared for histological examination in the same way there was no evidence that artefact played a part in the production of vacuolation.

In carrying out this histological examination, it was found that vacuoles were usually more numerous in the anterior two-thirds of the medulla than in the posterior one-third, and that a convenient point for sectioning was at a level just anterior to the calamus scriptorius, as suggested for sheep by Holman and Pattison (1943). Further, it was noted that vacuoles were particularly numerous in scrapie cases in the cells of the reticular formation, especially on or close to the median raphé. The rare vacuolated cells found in clinically healthy goats occurred in cells of the lateral and dorso-lateral reticular formation.

The appearance of vacuolated cells in the medulla oblongata of scrapie-affected goats is illustrated in Figs. 7, 8 and 9. Bubbly vacuolation (Figs. 7 and 8) was commonly noted, and in many cases there was enormous distension of the affected cell (Fig. 9). Vacuolation was frequently more intense than is usually seen in sheep. In addition to vacuolation of the neurones themselves, there was commonly vacuolation of the interstitial tissue of the medulla (Figs. 10 and 11). In some cases, groups of cells forming recognisable nuclei, e.g. the lateral cuneate, had disappeared and their position was marked only by holes in the tissue. The changes noted in many sections were like those of autolysis, but as all the tissues were placed in fixative within a maximum of three hours after death it was concluded that the abnormalities noted were not associated with post mortem decomposition. In addition to vacuolation of neurones in the medulla oblongata, degenerative changes of a more usual kind e.g. shrinkage, pyknosis, loss of staining power (Fig. 12) were also noted in many nerve cells in scrapie-affected animals.

It is to be emphasised that the figures for counts of vacuolated nerve cells in scrapie-affected goats given in Table 2 were obtained by examination of tissue from animals in a late stage of the disease. Examination of sections of the medualla oblongata from sheep slaughtered in the earliest clinical stage of experimental scrapie has indicated that vacuoles are less numerous than in a late stage of the experimental disease, and this was also the case in one goat that was

slaughtered when only the earliest clinical symptoms of scrapie were apparent.

DISCUSSION

If the apparent high degree of susceptibility of the goat to intracerebral inoculation of scrapie-infective material is confirmed by future observations, and the indications from experiments at present in progress are that this is likely, work on the disease would be considerably facilitated because certain observations could then be made on fewer experimental animals with greater certainty of reproducing the disease.

The presence of numerous vacuolated nerve cells in the medulla oblongata of all scrapie-affected goats and the difficulty, by contrast, of finding vacuolated nerve cells in the medulla oblongata of healthy goats, either non-inoculated or inoculated intracerebrally with brain suspension from a normal sheep, does much to answer the scepticism of Bosanquet, Daniel and Parry (1956) and Parry (1957) as to the relationship between nerve cell vacuolation and clinical evidence of scrapie. It has never been suggested, and we do not now suggest, that vacuolation of nerve cells is either the essential pathological lesion in scrapie or the cause of the clinical syndrome, but we have now added confidence in reaffirming that in the presence of clinical symptoms of scrapie the finding of vacuolated nerve cells is a most useful confirmation of diagnosis. It has been suggested by Bosanquet, *et al,* (1956) and by Parry (1957) that vacuolation of nerve cells is a relatively common finding in diseased human nervous tissue and in diseased and normal animal tissue; this has not been our experience in examining animal tissues, and we know of no other disease in sheep or goats in which vacuolation of nerve cells is constantly associated with a clear clinical syndrome, or in which massive nerve cell vacuolation can occur in a proportion of cases.

Since the first experimental transmission of scrapie by Cuillé and Chelle (1936) the experimental transmissibility of the disease to sheep has been demonstrated many times. However, the validity of these transmissions has recently been challenged by Bosanquet *et al.* (1956) who, while not themselves carrying out experimental inoculations, felt that the experiments of other workers had not been sufficiently controlled in that the sheep used might have come from "tainted" stock and might in any event have contracted scrapie. They expressed the opinion that "the precautions taken did not make it certain that the sheep used in these experiments were completely free from all forms of scrapie." We consider that if the additional evidence on the transmissibility of the disease to goats presented in this paper is added to that obtained from earlier work with sheep, there can remain no reasonable doubt that scrapie can be transmitted by experimental inoculation.

CONCLUSIONS

Intracerebral inoculation of five female and five castrated male goats with 1·0 ml. of brain suspension from a clinically normal Welsh Mountain sheep failed to produce disease during an observation period of 22 months.

Intracerebral inoculation of ten castrated male goats with 1·0 ml. of brain suspension from an experimental case of scrapie in a Welsh Mountain sheep produced scrapie in all animals within 22 months.

Subinoculations, using brain suspension from scrapie-affected goats injected by the intracerebral route, of ten female and 16 castrated male goats and 20 South Scotland Cheviot wethers produced scrapie in all the goats and in eight of the sheep. The period between inoculation and appearance of symptoms varied among goats from seven to 21 months, and among sheep from four to 11 months.

Third and fourth passages of scrapie were made through castrated male goats and 11 of the 12 animals developed scrapie within 12 months. At the time of writing 14 months after inoculation, the twelfth animal in these passages remains unaffected.

Clinical scrapie developed approximately 10 months after inoculation in all of three goats inoculated intracerebrally with 1·0 ml. of cerebro-spinal fluid obtained from a goat showing clinical evidence of scrapie.

The clinical appearance of goats affected with scrapie is described and attention is drawn to certain differences between the clinical appearance of the disease in the goat and that usually observed in the sheep.

Histological examination revealed large numbers of vacuolated nerve cells in the medulla oblongata of goats killed in an advanced clinical stage of scrapie; by contrast, vacuolated nerve cells were very rarely found in sections obtained from clinically healthy goats.

It is concluded that the high percentage of successful takes following intracerebral inoculation of goats with material from scrapie-affected sheep or goats, in contrast to a take in sheep that rarely reaches 60 per cent, may be of value in further experimental work on this disease.

ACKNOWLEDGMENT

We are most grateful to Mr. F. H. Summerfield, A.R.P.S., for preparing the illustrations.

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Figs. 4, 5, and 6. The clinical appearance of goats affected with scrapie following experimental inoculation.

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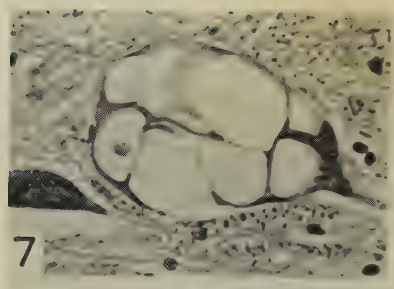

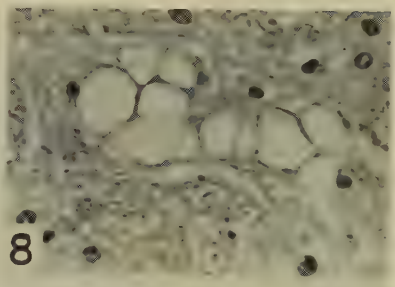

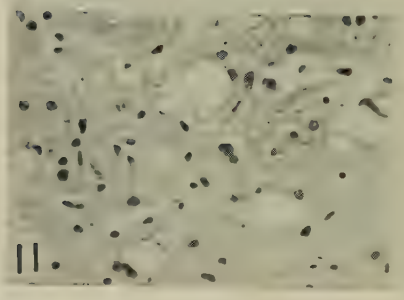

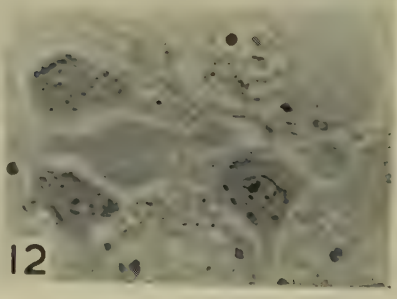

Figs. 7, 8, and 9. Vacuolation of nerve cells in the medulla oblongata of goats affected with scrapie. × 300.

Figs. 10 and 11. Vacuolation of interstitial tissue of the medulla oblongata in goats affected with scrapie. Fig. 10 × 80. Fig. 11 × 240.

Fig. 12. Degeneration of nerve cells frequently seen in the medulla oblongata of goats affected with scrapie. Fig. 12 × 240. All figures stained H. and E.

INFECTIOUS GASTRO-ENTERITIS OF PIGS

II. Transmission and Neutralization Experiments

By

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INTRODUCTION

During the second half of 1957 and the first quarter of 1958, a highly infectious type of gastro-enteritis spread among some of the pig herds of East Anglia. In a previous paper (Goodwin and Jennings, 1959), details were given of the clinical signs, pathology and epidemiology of this disease as it then occurred in the field. We now report work on the experimental reproduction of the infection, including the results of neutralization tests using serum prepared against the virus of Transmissible Gastro-enteritis, a similar disease in the United States.

MATERIALS AND METHODS

Preparation of infective material. The unfiltered suspensions were prepared from the intestinal contents and brain (experiment L6) or the intestinal contents alone (experiments RC and 132) of affected animals. These basic materials, as with all other infective samples, had been deep frozen until required in a cabinet containing solid carbon dioxide. In experiments L6 and 132, the material was ground and suspended in broth, centrifuged at about 2000 r.p.m. for 10 to 15 min. and the supernatant fluid collected. With experiment RC, the initial material was watery faeces, which after centrifuging without the addition of broth provided a sufficient volume of supernatant fluid.

The stock infective suspension used in the serum neutralization experiments was made by taking 30 g. of intestines with their contents and grinding with 270 ml. of broth. This intestinal material had been collected from three experimentally infected new-born pigs (litter 49) and deep frozen before use. The broth mixture was centrifuged at about 2000 r.p.m. for 10 min., when the supernatant fluid was removed and centrifuged again at about 8000 r.p.m. for 30 min. The resulting supernatant fluid was dispensed into glass phials that had been cleaned by a standard Calgon/metasilicate method. Except in the titration experiment, where a phial was used on the day of preparation, the phials were deep frozen together until required; they were then thawed rapidly and serially diluted with phosphate-buffer-saline (pH 7·2). Once diluted, the suspensions were incubated and administered as soon as possible. The dilution figures refer to the dilution of crude intestinal material, the phial contents already being at 10^{-1}.

The bacteria-free suspensions were all filtered through gradocol membranes and for brevity in the text the average pore diameters of the membranes used are given in parenthesis as each filtrate is mentioned. The filtrates were checked for bacterial sterility on blood agar. Apart from experiment HT, where a mixture of brain and intestine was used, the initial material was freshly-ground intestines and their contents or,

occasionally, intestinal contents alone. Such material was diluted between about 1:20 and 1:50 with broth and centrifuged at about 3000 r.p.m. for 10 min. The supernatant fluid was passed through a Seitz clarifying pad that had just previously filtered some serum-broth and this first filtrate was then taken through a gradocol membrane into a darkened flask. The final filtrate was used experimentally within an hour or so of collection.

Sera. Two types of immune sera were received from the United States. Serum 78 was from a pig that had been delivered by hysterectomy and kept in strict isolation. When 4 weeks old it was orally infected and then injected intramuscularly 10 days later. The antigenic suspension used was penicillin-streptomycin-treated intestine from a twelfth passage of Transmissible Gastro-enteritis virus; all the passages had been in colostrum-free piglets delivered by hysterectomy. The serum was harvested 21 days after the injection and inactivated at 56°C. for 30 min. No preservative was added and storage in the United States had been at —20°C. Serum 33 was prepared in a pig that had not been so rigidly isolated before infection and this reagent, therefore, could be less specific. When tested in the United States according to the technique of Young, Underdahl and Hinz (1953) serum 78, diluted 1:8, neutralized 1000 ID_{50} intranasal doses; serum 33, when diluted 1:16, similarly neutralized these infective doses. In our neutralization experiments, 1 ml. of imported serum was incubated with 1 ml. of infective suspension for 1 hour at 37°C. and all of the mixture was given by mouth.

The serum used for prophylaxis, both experimentally and in the field, was obtained from a group of bacon pigs slaughtered about 3 weeks after being affected with profuse diarrhoea in a typical herd outbreak. It is referred to as convalescent serum and was inactivated at 56°C. for 30 min. before use.

Pigs. The experimental herd in which nearly all the various transmission experiments were made consisted of about 40 sows with their associated litters and fattening pigs. This herd was largely home-bred and no condition resembling gastro-enteritis had been seen before the disease was deliberately introduced.

The colostrum-free piglets used in the titration and neutralization experiments were produced as described by Betts, Lamont and Littlewort (1959). When newly-born they were kept in incubators in a building especially designed to house highly-infective diseases. Before dosing, they were moved from the incubators to individual rooms in the same building. These rooms were strictly isolated and were each entered from an anteroom where overalls, boots and gloves were donned.

RESULTS

Experimental Transmission of the Disease

Transmission by Direct Contact

The disease was transmitted very readily to suckling pigs by direct contact. On the first occasion, two ailing piglets, 9 days old, were brought to Cambridge from an affected herd. They were substituted for two other piglets in a litter of eleven that were 5 days old. One of the in-contact pigs died 3 days later, a second died

after 4 days and three more after 5 days; both the original piglets also died. The four survivors were early-weaned and retained. About a week later the experiment was repeated in two other litters that were housed about 300 yards away and attended by a separate pigman. Two affected piglets, 2 days old and from the same field outbreak, were added to a litter of ten that were 8 days old. Both these piglets died between 24 and 48 hours later, by which time the in-contact piglets had developed diarrhoea. Eighteen hours after the introduction of the natural cases, two in-contact piglets were removed and added to a second litter of ten that had been born the previous day. The transferred piglets appeared healthy when moved, but evidently they were then incubating the disease because they showed diarrhoea 24 hours later and, once again, the in-contact piglets in the same pen became affected. All the survivors of the two litters were destroyed at this stage to try to prevent further spread of infection.

Transmission by Indirect Contact

In both the separate groups of buildings where the above experiments had been made the disease soon spread to other litters without any direct contact. Neither of the buildings was suitably designed to prevent the spread of highly infective conditions, but in the one where the first experiment had been made the four surviving piglets were in a separate concrete box outside which was a tray of disinfectant; the attendant understood that it was hoped to confine the infection to the one litter. However, in this block and the adjacent block there were five other unweaned litters and all of these rapidly contracted the disease. In the groups of buildings where the disease was introduced for the second time, a sow farrowed ten piglets 4 days later and the whole litter died within a few days. It was clear that simple precautions and routine disinfection of boots had been inadequate to contain the disease in both instances. The condition returned several times in different parts of the herd during the ensuing months but, by then, indirect transmission was no longer the only possible route of infection, because many animals had been moved about within the herd.

Transmission by Unfiltered Material

Two experiments were made using unfiltered material alone. In the first (L6), five pigs in a litter of nine were infected orally when 8 days old. By 40 hours three of the five had diarrhoea; by 48 hours two of the four in-contact piglets had diarrhoea and by 60 hours the whole litter was affected. The diarrhoea started to resolve at 4 days and had completely cleared by 7 days after infection. One of the piglets died 5 days after being dosed and the lesions were typical of a severe field case. A sow with a litter 12 days old had been placed in the adjacent pen at the time of dosing and no precautions were taken against the spread of infection. Despite

this, the disease did not extend beyond the infected litter and hence both the severity of the disease and its apparent infectivity appeared to have diminished since the original introduction of the causal agent into the herd about 2 months previously.

The experiment was then repeated using two young weaners brought in from a farm (RC) where the disease was unknown. A different sample of infective material was prepared and the pigs were housed on completely separate premises 3 miles away. One pig was dosed by mouth and the other was left in contact; it having been previously injected with 50 ml. of serum collected from a convalescent bacon pig. The unprotected pig suffered from profuse diarrhoea and inappetance, but the other animal showed no sign of infection at any time.

Transmission by Bacteria-free Filtrates

Gradocol membranes of A. P. D. 0·67μ or more. In the first of these experiments (HT), three of five piglets were dosed orally and injected subcutaneously on the second day of life (filtrate, A.P.D. 0·67μ). On the following day two of the dosed animals had slightly loose faeces, but this was transient and no other signs developed. The experiment was repeated using a filtrate prepared in the same way from the same sample of preserved material. Eight piglets (litter 132) were either injected subcutaneously or dosed by mouth on the second day of life and the other five in the litter were left in contact. Three days later, two piglets from each of the treated and in-contact groups were dead, but none of these deaths could be ascribed to infection. As the six other dosed piglets remained healthy, they were re-infected orally with aliquots of the same filtrate, which had been kept deep frozen. Once more, there were no ill-effects and the faeces of all the animals remained firm. It now appeared that such filtrates could not initiate the disease under these conditions and, to ascertain whether the failure was due solely to the process of filtration, the six treated piglets were challenged on the 12th day of life with the supernatant fluid from which the filtrate had been prepared. Although the dosed animals were by now much older, a watery diarrhoea developed in two of them 24 hours later, and by 72 hours the whole litter, including the three in-contact piglets, were similarly affected. One of the dosed piglets was killed at this stage but, apart from a catarrhal enteritis, it showed no gross pathological changes. The remainder progressively recovered and were all clinically normal 8 days after infection.

One more attempt was made to infect a litter of piglets with filtered material of this type. Two different sources of infective material, not previously used, yielded two separate filtrates (A.P.D. 0·79μ) and the latter were fed to two groups of three piglets, respectively, in a litter of eleven, 5 days old. One of the dosed piglets had fluid faeces during the 4th day after infection but, otherwise, the litter remained normal.

A likely reason for these largely negative results could have been that the infected litters had gained some degree of passive immunity from their dams, and hence a similar experiment was performed with pigs that had been reared artificially in a manner similar to those used in the neutralization experiments. Two pigs, 14 weeks of age, were dosed by mouth with 5 ml. of filtrate (A.P.D. 0·81μ); two litter-mates were left in contact. There was a marked reaction by 36 hours, when both the infected pigs were dull, not eating and suffering from diarrhoea. The next day, these two pigs were still not eating, both had profuse diarrhoea and their rectal temperatures were 105° and 106° F., respectively. By this time one of the in-contact pigs was not eating and on the following day both the in-contact animals had rectal temperatures of over 105°. Each pair of animals recovered in about 1 week from the first clinical signs.

Gradocol membranes of A.P.D. 0·32μ. The influence of passive immunity in preventing experimental infection appeared to be confirmed by the last experiment and, as a result, five heavily in-pig gilts were obtained from a farm where the disease had not appeared, to see whether their litters would be more susceptible. Until each gilt was about to farrow it was held on adjacent premises and attended by a separate pigman.

When the first gilt farrowed it was kept on the holding premises until the litter was 10 days old; the gilt was then moved to the usual experimental accommodation and three out of her nine piglets were dosed orally with filtrate (0·32μ). The experiment was completely negative. Shortly afterwards a litter was farrowed by a sow in our experimental herd and three out of five piglets were dosed orally on the second day of life with a similar filtrate. Only one of the treated animals showed clinical signs of infection; this pig had a watery diarrhoea 4 days later and died after 8 days with widespread pathological changes typical of the natural disease.

The last two experiments suggested that the litters of the purchased gilts would probably be no more vulnerable than those in our own herd; in both cases piglets might be successfully infected but it was impossible to forecast when or to what extent. It was decided, therefore, to infect future piglets before they had suckled.

The second purchased gilt (D2) farrowed a litter of seven, five of which were removed at birth. The infective filtrate (0·32μ) was given orally to three animals when they were under 5 hours old and the two other colostrum-free piglets were injected subcutaneously with 15 ml. of convalescent serum. These five piglets were returned to the sow 24 hours later; they were then lively and soon suckled well. By 48 hours after infection all three dosed piglets had a watery diarrhoea and by 72 hours all but one animal in the litter were dead. The sole survivor, one of the animals given serum, was badly affected; it was dead by the following day. Both the piglets injected with serum and the dosed animals showed pin-point gastric haemorrhages and enteritis, even though the disease had been terminated

early in its course. This rapid death of the litter was hastened by the sow; she also contracted the infection, 48 hours after contact with the dosed piglets. Developing profuse diarrhoea and complete anorexia, she had no interest in her piglets and these, being already ill, were soon overlain. It is noteworthy that this sow was the only one to contract the disease clinically throughout all the transmission experiments.

The experiment was repeated with a litter of three, born from a sow (49) in the experimental herd. The filtrate (0·32μ) was prepared from the intestinal contents of one of the dosed pigs in the above litter (D2); this, therefore, was the second serial passage. The piglets were removed before suckling, dosed orally within 3 hours of birth and returned to the sow when about 24 hours old. Forty hours after infection all three piglets had a watery diarrhoea and they died between 48 and 60 hours after being dosed. The changes seen post mortem were mainly of marked enteritis, but one animal had gastric haemorrhages. The intestinal contents from these three piglets were pooled and used to provide a stock infective suspension for the serum neutralization experiments described later. It was now clear that by removing the piglets at birth, and thereby delaying access to the colostrum, the disease could be readily induced.

Serum Neutralization Experiments

Before using the sera prepared in the United States against Transmissible Gastro-enteritis, our stock infective suspension was titrated in colostrum-free piglets. A litter was orally infected with 2 ml. quantities of serial dilutions of the suspension when 9 days old and the main findings are summarized in Table 1. It will be seen that there was a consistent graded effect, from death to a complete absence of clinical signs.

TABLE 1

TITRATION OF INFECTIVE AGENT

Effect of serial dilutions given orally to colostrum-free piglets

Number of piglets	*Dilution of infective suspension*	*Remarks*	*Outcome*
2	10^{-5}	Both ailing at 36 hrs. Diarrhoea and vomiting.	1 died at 84 hrs. 1 died at 108 hrs.
3	10^{-6}	All ailing at 36 hrs. Both survivors had watery diarrhoea.	1 died at 60 hrs. 2 survived.
2	10^{-7}	Only one pig affected; diarrhoea at 48 hrs.	2 survived.
2	10^{-8}	Faeces remained firm throughout.	2 survived.

A protection experiment was then performed using serum 78. Eight piglets were healthy with firm faeces and a ninth was weak and affected with diarrhoea; it was held in reserve, therefore, and four groups of two piglets were dosed orally when 8 days old (Table 2). It should be recalled that 1 ml. doses of suspension were given in this and the subsequent experiment. After 96 hours all four groups were completely healthy, and challenge experiments were initiated, the litter then being 12 days old (Table 2). Before this, however, the reserve piglet had strengthened and developed firm faeces; it was infected orally with a 10^{-4} suspension plus buffer when 11 days old, and, having developed profuse diarrhoea 24 hours later, it died after 5 days. Only one piglet of those challenged with suspension alone in the four main groups survived; this animal was badly affected, but by now age resistance could have influenced the outcome considerably. The single death in the group receiving serum did not appear clinically to be due to gastro-enteritis as neither piglet had developed diarrhoea by 96 hours. The post-mortem examination revealed widespread mottling of the liver, which histologically proved to be telangiectasis; there was no sign referable to gastro-enteritis apart from a mild catarrhal enteritis.

In a third experiment, the protective effect of sera 78 and 33 were compared. A litter of nine was infected when 5 days old (Table 3). As they came out of the incubators at a younger age, most had diarrhoea when the experiment started. Of the positive controls, the piglet given the 10^{-4} dilution died, while the one given the 10^{-5} lived. To confirm the lethality of the former dilution, the surviving control was challenged with a 10^{-4} suspension and it also died.

Epidemiological and Pathological Observations

The disease was first consciously introduced into the experimental herd in November, 1957, and from early-January to late-April, 1958, a series of transmission experiments was made on the same premises. Despite this, the infection has at no time become an acute problem as seen in the field and only one sow, which had been recently purchased, contracted the disease. On the other hand, the disease has been a recurrent problem among the young piglets and this aspect will be described in more detail.

Evidence that the infection could spread somewhat insidiously came from a litter that had been experimentally infected when 8 days old. At 2 weeks of age their recovery appeared complete and, when weaned 6 weeks later, they were moved to a fattening pen. After 1 more week, a normal newly-weaned litter was placed in the same box and several of these pigs became very unthrifty. One of the experimentally-infected pigs died when nearly 11 weeks old and a second when 13 weeks old, at which time four of the unthrifty in-contact pigs were sacrificed. All these animals showed a chronic enteritis, as did a seventh pig 5 weeks old dying 6 months later, and

TABLE 2

FIRST SERUM-NEUTRALIZATION EXPERIMENT

Neutralization and challenge experiments in colostrum-free piglets using serum 78

Number of piglets	*Dilution of infective suspension*	*Remarks*	*Challenge material*	*Remarks*	*Outcome*
2	10^{-5} + serum (78)	No effect by 96 hrs.	10^{-5} + buffer	Both affected with diarrhoea after 96 hrs.	Died after 5 and 7 days, respectively.
2	10^{-4} + serum (78)	,,	10^{-4} + buffer	Both affected with diarrhoea after 96 hrs.	Died after 4 and 9 days, respectively.
2	10^{-3} + serum (78)	,,	10^{-3} + buffer	Both vomiting and diarrhoea after 48 hrs.	One died after $3\frac{1}{2}$ days; other survived.
2	10^{-2} + serum (78)	,,	10^{-2} + serum (78)	Both firm faeces throughout.	One died after 4 days (see text); other remained healthy.

in three pigs there was a chronic gastritis, with a grossly thickened and folded mucous membrane, excess mucus and, in one instance, a firmly-adherent lining of biscuit-coloured material. The significant lesion in all, however, was in the terminal portion of the ileum. Here the mucosa was corrugated, much like the ridging seen in Johne's disease in cattle, and covered with a cheesy necrotic material. The hose-pipe thickening in the region of the ileo-caecal valve almost obliterated the lumen (Fig. 1). Histological examination showed extensive superficial necrosis and a diphtheritic mucosa. There was a good deal of cellular granulation tissue, and infiltration of the mucous membrane and submucosa with plasma cells and macrophages (Fig. 2). This resulted in obliteration of the mucosal glands. No lesions such as perivascular cuffing, gliosis or neuronal degeneration were seen in any of these chronic cases.

Further evidence for the persistence of the infection in a chronic form, with occasional mortality among the unweaned piglets, was obtained during 1958. In May, a whole litter of fourteen died within a few days of birth, nine out of thirteen died in a second litter and seven out of ten in a third. Also in May, two piglets in a litter of nine showed clinical signs and died with typical lesions, which demonstrated that the disease might attack not only occasional litters but one or two piglets in otherwise healthy litters. This pattern of fitful emergence of the disease among a steady succession of normal litters has continued until the present time, 5 months after the last experimental introduction of infective material. In the field, where it is not the practice to investigate all spasmodic and isolated deaths, cases such as these could well be ascribed to other causes.

One litter was infected experimentally in order to observe the early development of the lesions. The unfiltered infective material was prepared by centrifuging and taking the supernatant fluid from the intestinal contents of one of the piglets that died in the titration experiment; it was, therefore, the fourth serial passage. One piglet was killed within 1 hour of birth, as a control, and three others were deprived of colostrum until infected about 5 hours after birth. Of the latter animals, one was killed 12 hours after infection, not having suckled at all; the other two were returned to the sow 16 hours after infection and killed at 36 and about 50 hours after infection, respectively. There were no significant macroscopic changes in the first two infected animals but, histologically, both showed lesions. The earliest change, at 12 hours, was congestion of the mesenteric vessels with an increased activity of the mesenteric lymph nodes, the chief feature being the very large number of mitotic figures. At 36 hours there was considerable submucosal oedema, the mucous glands of the intestine were very active and the tips of the villi were showing degenerative changes. Around the villi was much eosinophilic albuminous material and the intestinal epithelial cells were very granular. Polymorphonuclear leucocytes were present in the tips of

TABLE 3

SECOND SERUM-NEUTRALIZATION EXPERIMENT

Neutralization and challenge experiments in colostrum-free piglets using sera 78 and 33

Piglet	*Dilution of infective suspension*	*Remarks*	*Outcome*	*Challenge material (given at 4 days)*	*Remarks*	*Outcome*
8	10^{-4} + buffer	Diarrhoea by 48 hrs., weak by 72 hrs.	Died by 96 hrs.			
9	10^{-5} + buffer		Lived	10^{-4} + buffer	Diarrhoea 2 days later.	Died 6 days after challenge.
1	10^{-4} + serum (33)	Watery diarrhoea by 72 hrs.	Died by 96 hrs.			
2	10^{-3} + serum (78)	Weak at start of experiment.	Died at about 24 hrs.			
3	10^{-3} + serum (33)		Lived	10^{-1} + buffer	Vomited 2 days later.	Died 5 days after challenge.
4	10^{-2} + serum (78)		Lived	10^{-1} + serum (33)	Diarrhoea 2 days later.	Died 4 days after challenge.
5	10^{-2} + serum (33)	Weak by 24 hrs.	Died by 48 hrs.			
6	10^{-1} + serum (78)		Lived	10^{-1} + serum (33)	Diarrhoea 2 days later.	Died 5 days after challenge.
7	10^{-1} + serum (33)	Diarrhoea by 72 hrs.	Died by 5 days.			

the villi and the capillaries of this area were acutely congested. The proximal convoluted tubules of the kidney showed degenerative changes and many were already occluded. There were no changes in the central nervous system and meninges. The last piglet, killed at 50 hours, showed slight gross lesions, namely, enteritis and congestion of the mesenteric vessels. Histologically, there was necrosis of many villi and considerable leucocytic activity. These intestinal changes were more severe than the gastric changes. The renal damage was severe; not only were the tubular lumina occluded but the glomeruli were much swollen and excessively nucleated.

DISCUSSION

In six normal litters dosed with infective suspensions there was little clinical evidence of disease and only two deaths. In sharp contrast to this, all the piglets that were infected before suckling in three other normal litters contracted the disease and, apart from two that were killed, they all died. It is clear, therefore, that passive immunity greatly influences the outcome of infection, although there is probably a balance between the immunity that can be derived from the colostrum and the intensity of infection. Thus continual exposure to direct-contact infection was able to overcome the natural passive immunity of new-born piglets, and in one litter where a suspension that had passed through a gradocol membrane (A.P.D. 0·67μ) was twice completely ineffective, all the animals developed diarrhoea when challenged with the same material unfiltered, even though they were then several days older. A similar variation in susceptibility to Transmissible Gastro-enteritis was noticed by Doyle and Hutchings (1946) and Feenstra, Thorp, Gray and McMillen (1948). Convalescent serum injected subcutaneously before infection, however, did not appear to be a substitute for colostrum. Although a weaned pig (experiment RC) given serum withstood in-contact infection, this animal may have been naturally immune, and the deaths of the protected pigs in litter D2 were more in keeping with experience in the field, where the limited use of convalescent serum has lent no support to the idea that such serum might be useful in preventing infection among new-born litters. The effectiveness of colostrum probably derives from the fact that natural suckling provides a continual source of antibody within the alimentary tract.

The observations on this disease in our experimental herd may throw light on the pattern of the disease in the field. In the most dramatic natural outbreaks, many of the adult and fattening pigs become affected and in these herds there is probably little natural immunity. In other herds, such as ours, the adult and fattening stock appear quite resistant and they may act as a barrier, greatly reducing the risk of natural infection. A similar situation exists with herds vaccinated against swine fever; although the suckling

litters are vulnerable it is unusual for swine fever to become established among them, whereas a completely unvaccinated herd is very much more liable to infection. It is interesting that the natural immunity of our own adult stock was not associated with any previous evidence of infection and a similar immunity could be widespread among British herds. Once the disease enters a largely-immune herd, however, it can persist for many months, periodically flaring up and killing new-born piglets. One of our litters (L6) was infective to other pigs 7 weeks after its clinical recovery and the long-standing enteritis in these animals suggests that their faeces were the main means of disseminating the disease. Lee, Moro and Baker (1954) were able to demonstrate the virus of Transmissible Gastro-enteritis in the faeces of pigs 8 weeks after infection. To what extent other herds may be carrying the infection in this chronic form is not known but there could be a number of such smouldering foci from which, under appropriate conditions, future acute outbreaks could arise.

The chief lesions seen in piglets dying from this disease in the field have been varying degrees of gastro-enteritis and meningeal congestion. The gastric changes range from pin-point haemorrhages to ulceration, and the intestinal changes vary from a mild catarrhal to a peracute haemorrhagic enteritis (Goodwin and Jennings, 1959). Although normal piglets dying from experimental infection showed the same range of gross and microscopic changes, those infected after hysterectomy did not usually show such advanced lesions, even though they were mostly 2 weeks old. In only 25 per cent of the latter cases was the enteritis haemorrhagic; the gastritis also was generally much less acute and there was never ulceration. A similar lack of well-developed lesions has been observed in colostrum-free pigs affected with Transmissible Gastro-enteritis (Young, Hinz and Underdahl, 1955) and it is probably due partly to the disease taking a more rapid course in the complete absence of protective antibodies and partly to the reduced influence of secondary bacteria. Histologically, there are two points worthy of comment. First, although meningeal congestion was a common finding in all types of experimental infection, no case of encephalitis was encountered, either in the young pigs or in the animals believed to be chronic cases of the disease. Secondly, the renal changes were so severe that they must make a serious contribution to the cause of death. Almost all the proximal convoluted tubules were commonly occluded by necrotic and swollen epithelial cells, and this occlusion was quite marked 36 hours after infection.

The thickened ileum seen in the seven chronic cases of enteritis resembled in its gross and microscopic pathology the condition of Terminal Ileitis described by Field, Buntain and Jennings (1953). There were no specific giant-cell systems, as seen in Crohn's disease in man, and none of the anaplastic changes in the superficial and glandular epithelium described by Emsbo (1951) in the pig.

The distribution of the lesion may be related to the anatomy of the ileo-caecal junction, where there is both a natural obstruction and a marked congregation of submucosal lymphatic tissue, which could concentrate infective agents. The type of thickening seen in our cases, therefore, and previously described as Terminal Ileitis, may not be a specific entity, but the end-result of a variety of chronic intestinal irritations. A similar conclusion was reached by Emsbo (1951) reasoning in the reverse direction from his cases of Terminal Ileitis.

As stressed elsewhere (Goodwin and Jennings, 1959), the similarity between field outbreaks of the disease now described and Transmissible Gastro-enteritis is virtually complete. This similarity has continued with the experimental work and the serum-neutralization tests suggest that the causal agent might be the same. In the first neutralization experiment, it could be argued that, as no piglets contracted the disease initially, the first phial used was not infective. This is improbable, as the next four phials taken from the same box were all satisfactory and, as a 10^{-5} suspension was still lethal, complete protection against a thousand lethal doses (10^{-2}) had almost certainly been achieved. In the challenged animals there was definite protection against the 10^{-3} suspension, the lowest dilution tried. In the second neutralization experiment, the protective effect of serum 78 was clear-cut, but for the death of Pig 2. This animal was very weak at the outset and it might well have died even if it had not been dosed. Once again, therefore, serum 78 protected against a thousand lethal doses. With serum 33, however, the results were inexplicable, since this serum should have been equally protective. There was no protection against the 10^{-1} and 10^{-2} suspensions and it is difficult to assess the findings with the other dilutions. In interpreting both the main neutralization experiments, it is useful to note that whenever piglets were given a sufficient dose of suspension alone they contracted the infection; furthermore, all the piglets successfully protected with serum, and subsequently challenged, were shown to be susceptible.

CONCLUSIONS

A highly infective form of gastro-enteritis in pigs has been transmitted experimentally by direct and indirect contact. The disease has also been reproduced with bacteria-free filtrates prepared from the faeces of affected animals.

Normal piglets were relatively resistant when dosed with infective suspensions but proved highly susceptible if they were denied colostrum beforehand.

A chronic form of enteritis was produced in which the most consistent finding was gross thickening of the ileum. This lesion closely resembled the condition previously described as Terminal Ileitis.

Up to four serial passages were made with the infective agent, which passed through gradocol membranes of A.P.D. 0·32μ and appeared to be specifically neutralized by a serum (78) prepared in the United States against Transmissible Gastro-enteritis.

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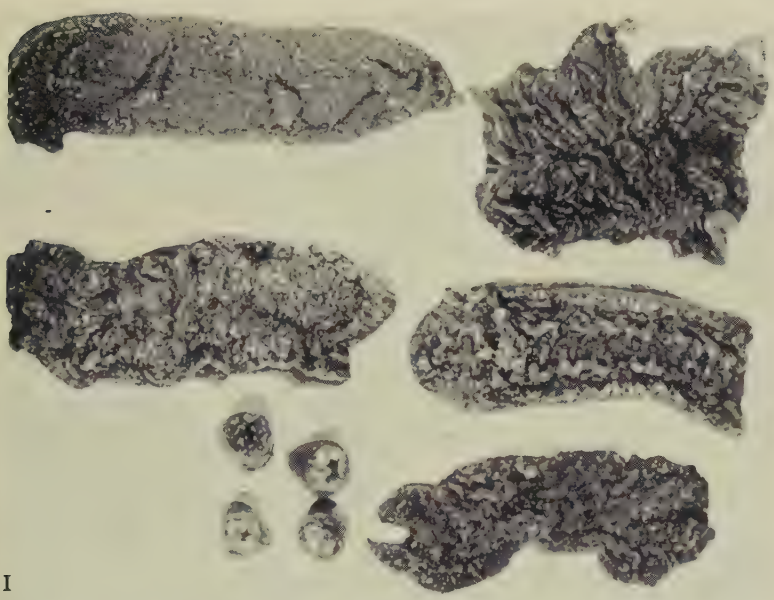

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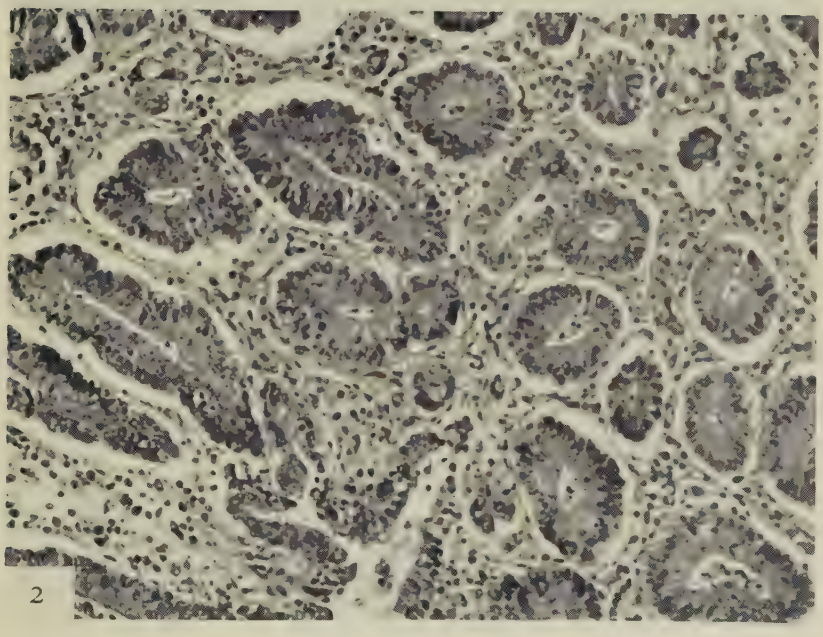

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Fig. 1. Portions of intestine from a 11-week-old pig, showing corrugation and necrosis.

Fig. 2. Section through mucosa of ileum from a 13-week-old pig, showing infiltration with mononuclear cells. × 230.

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ANTIBIOTICS IN CHEMOTHERAPY OF CAECAL COCCIDIOSIS IN CHICKENS*

By

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INTRODUCTION

The effects of small quantities of certain antibiotics in poultry food are well known, and their mode of action in promoting growth has received much attention. In this connection the activity of antibiotics against specific infections may be important.

Seeger, Lucas and Tomhave (1950) reported good control of caecal coccidiosis by using aureomycin at approximately 0·0125 to 0·02 per cent in the food. The anticoccidial activity of this antibiotic has also been recorded by Waletzky (1951), Marthedal and Velling (1953), Kostenko (1955), Cuckler and Malanga (1957) and Dzhilavyan (1957). Blount (1954) obtained good results using aureomycin at approximately 0·005 per cent in combination with nitrophenide in outbreaks of intestinal coccidiosis, as did White-Stevens, Zeibel and Smith (1955) with aureomycin at 0·005 to 0·02 per cent mixed with various coccidiostats under field conditions. Gardiner (1957) found that the combination of aureomycin and sulphadimidine was more effective in suppressing caecal coccidiosis than either compound used alone at the concentrations examined. Of the twelve antibiotics examined by Cuckler and Malanga (1957) only spiramycin, apart from aureomycin, showed good activity against experimental *Eimeria tenella* infections. They found that penicillin and streptomycin, which at low concentrations had previously been found inactive (Todd, Wyant and Elam, 1951; Wilson, 1953), proved only slightly effective at 0·5 per cent in the food. On the other hand, Nikolsky and Zolotovsova (1955) claimed that the administration of penicillin to chickens with coccidiosis had a beneficial effect.

The purpose of this paper is to report the results obtained with aureomycin, chloramphenicol, erythromycin, penicillin, spiramycin, streptomycin and terramycin against experimental infections of *E. tenella* in chicks.

METHODS

Rhode Island Red x Light Sussex cockerels from a single commercial hatchery were used throughout and isolated as far as possible from extraneous infections with coccidia before being placed on test. All experiments were carried out in wire-floored cages housed in an experimental room which was maintained at a temperature of approximately 90°F.

Aureomycin hydrochloride, chloramphenicol, erythromycin base, crystalline potassium penicillin G of a potency of 1,500 units/mg., spiramycin base and sulphate, streptomycin sulphate of a potency of 720 units/mg., and terramycin hydrochloride were used mixed in the food. Spiramycin (base and sulphate) was also examined in solution in comparison with sodium sulphaquinoxaline.

*The greater portion of this work formed part of a thesis for the M.Sc. degree of the University of London (1957).

A ten per cent w/w premix of each antibiotic was first prepared in plain wheat flour by thoroughly mixing with a pestle and mortar. From this the medicated food of required concentrations was made by mixing in a 'Kenwood Major' electric mixer with a proprietary baby chick mash. The spiramycin and the sodium sulphaquinoxaline solutions were prepared fresh daily. Spiramycin base was dissolved in absolute ethanol before diluting with tap water and no precipitation was noted during the following twenty-four hours. The drinking water containing the spiramycin sulphate was prepared by diluting a one per cent w/v aqueous solution of this salt.

The medicated food and drinking water were given to chicks at various times before or after infection which was accomplished, except where otherwise stated, by inoculating orally each chick when 7 or 8 days old with approximately 200,000 sporulated oocysts of the *E. tenella* strain originally obtained from Dr. C. Horton-Smith of the Houghton Poultry Research Station. In any one experiment the groups of chicks were balanced for weight. All treatments were terminated on the seventh day after infection when surviving birds were killed and examined, unless required for immunity tests or observation for any relapses. The majority of untreated infected chicks died on the fifth day following ingestion of oocysts and all birds were examined post mortem to confirm the cause of death.

Two criteria of activity were used: (a) the proportion of surviving chicks on the seventh day after infection, and (b) the severity of caecal lesions. The lesions were classified as follows : 0=no macroscopic lesions; 1=less than ten pin-point haemorrhages per caecum, often accompanied by blood or a small caseous core in the lumen of the caecum; 2=moderate number of haemorrhages, often with enlargement of the caeca, blood or a large caseous core in the lumen; 3=very severe haemorrhages with caeca distended with blood. These numerical values were used in computing averages.

RESULTS

Effect of Commencing Medication 24 or 48 Hours before Infection

The results of all experiments in which medication was started 24 hours before infection are summarised in Table 1. Unmedicated uninfected chicks were included in each test and these never showed caecal lesions at post mortem. In several experiments nicarbazin (0·02 per cent) nitrofurazone (0·02 per cent) or sulphaquinoxaline (0·05 per cent) were included to test that the infection was reacting normally and it was found that all three substances gave good control. Spiramycin (as base or sulphate) showed anti-coccidial activity when given in solution from 2 days before to 7 days after infection; the mortality was 4/10 with the sulphate at 0·025 per cent in the drinking water, compared with 1/10 on the same concentration of sodium sulphaquinoxaline, when 9/10 infected control chicks died.

Effect of Commencing Medication after Infection

These results are shown in Table 2. The total deaths in the

untreated infected controls were 53/60. Aureomycin, chloramphenicol and spiramycin prevented deaths when medication was not delayed for longer than 2 days after infection. Erythromycin had little effect whereas terramycin showed activity at the maximum concentration tested.

An uninfected group of 10 chicks fed 0·2 per cent spiramycin (base) increased their weight by 116 per cent from the first to the sixth day of medication, compared with 94 per cent for uninfected unmedicated controls. The infected group given 0·2 per cent spiramycin from 24 hours after infection passed no blood and gained 107 per cent in weight, and those starting medication 48 hours after infection gained 74 per cent. Spiramycin in the drinking water appeared more effective in controlling established infections than when given in the food (Table 3).

TABLE 1

ANTIBIOTICS IN THE FOOD FROM 1 DAY BEFORE UNTIL 7 DAYS AFTER INFECTION

Compound	*Treated groups*			*Untreated controls*	
	% in food	*Mortality*	*Avg. grade caecal lesions*	*Mortality*	*Avg. grade caecal lesions*
Aureomycin	0·2	0/10	0	19/20	2·9
	0·1	0/10	0·8		
	0·05	4/20	1·8		
	0·025	19/20	3·0		
Chloramphenicol	0·2	0/10	1·5	19/20	3·0
	0·05	10/10	3·0		
Erythromycin	0·3	2/10	2·2	19/19	3·0
	0·2	0/10	1·6		
	0·1	4/10	2·4		
Penicillin	0·2	8/10	2·8	9/10	2·8
Spiramycin (base)	0·3	0/10	0	38/39	3·0
	0·2	0/10*	—		
	0·05	0/10	1·0		
	0·025	0/30	1·0		
	0·0125	5/30	1·7		
	0·006	14/20	2·5		
Spiramycin (sulphate)	0·025	0/10	—	9/10	—
	0·0125	1/10	—		
Streptomycin	0·2	9/10	2·9	9/10	2·8
Terramycin	0·2	0/10	0·1	18/20	2·9
	0·05	4/10	—		
	0·025	6/10	2·4		

*No clinical symptoms of disease observed when drug-diet was replaced by unmedicated food from the 7th to the 13th day following infection.

Effect of Medication upon the Development of Immunity

Aureomycin, chloramphenicol and terramycin. Ten surviving chicks fed 0·1 per cent aureomycin, eight fed 0·1 per cent chloramphenicol and six survivors from 0·05 per cent terramycin were given normal diet from the 7th day after infection. Seven days later these chicks and ten previously unmedicated and uninfected birds of the same age and reared under the same conditions were each inoculated with approximately 400,000 sporulated oocysts of *E. tenella.* No deaths occurred in any of the previously medicated and infected groups and the majority showed no macroscopic caecal lesions on the 7th day following the 'challenge' infection. Four of the ten control birds succumbed to acute caecal coccidiosis, the remainder showing severe caecal lesions.

TABLE 2

ANTIBIOTICS IN THE FOOD FROM 1–3 DAYS AFTER INFECTION AND RESULTS DETERMINED 7 DAYS AFTER INFECTION

Compound	*Treated groups*		
	% in food	*No. days treatment started after infection*	*Mortality*
Aureomycin ..	0·2	2	0/10
		3	3/10
	0·1	2	0/10
	0·05	2	2/9
Chloramphenicol ..	0·2	2	0/10
		3	5/10
	0·1	2	2/10
	0·05	2	8/10
Spiramycin (base) ..	0·2	1	0/10
		2	0/10
		3	6/10
	0·1	1	0/10
		2	3/10
	0·05	1	0/10
		2	7/10
Erythromycin ..	0·2	2	7/10
Terramycin	0·1	2	2/10
		3	8/10
	0·05	2	6/10
Sulphaquinoxaline ..	0·05	2	0/20
		3	0/10

Spiramycin. In one group of 10 chicks fed 0·2 per cent spiramycin (base) from 1 day before infection, all chicks survived. Another group of 10 (Group A) was not infected but received the same medicated food. These chicks and those of two infected groups which received spiramycin 1 and 2 days after infection were all given

unmedicated food after 7 days. Fourteen days later they and another ten (Group B, uninfected and unmedicated) were each inoculated with approximately 400,000 sporulated oocysts of *E. tenella*. This 'challenge' dose proved only fairly severe, killing four chicks of Group A and three of Group B. No deaths occurred in any of the previously infected and treated groups. On the eighth day after the challenge infection the surviving birds were killed and very extensive caecal lesions and haemorrhages were found in Groups A and B, whereas the caeca of the majority of the previously infected and treated chicks appeared normal.

TABLE 3

SPIRAMYCIN (BASE) AND SODIUM SULPHAQUINOXALINE IN THE DRINKING WATER 2 DAYS AFTER INFECTION AND RESULTS DETERMINED 7 DAYS AFTER INFECTION

Group	*% in drinking water*	*Mortality*	*Average grade caecal lesions*
(Uninfected control)* ..	—	0/10	0
(Infected control)* ..	—	9/10	2·9
Spiramycin (base)† ..	0·2	0/10	0·1
	0·1	0/10	0·2
	0·05	2/20	1·1
	0·025	3/10	2·0
	0·0125	6/10	2·5
Sodium sulphaquinoxaline	0·05	0/10	1·5
	0·025	1/10	1·5

*Given 2% ethanol in drinking water from 2 days after infection.
†Medicated drinking water prepared by diluting a 10% alcoholic solution of spiramycin.

A confirmatory test was carried out using three groups of ten 7-day-old chicks. Group 1 was given a diet containing 0·2 per cent spiramycin for eight days beginning one day before infection. Group 2 was infected but received unmedicated food, and Group 3 was reserved as a control to the 'challenge' dose. Eight of the ten chicks in Group 2 died from acute caecal coccidiosis and all Group 1 survived. Fourteen days after the last death in Group 2, the chicks in Groups 1 and 3 were each given 400,000 sporulated oocysts. None of Group 1, but five of the ten chicks of Group 3 died. Seven days after the 'challenge' inoculation the survivors were killed and the five chicks remaining from Group 3 showed extensive caecal lesions, but the caeca of seven chicks of Group 1 showed no macroscopic lesions, the other three having small caecal cores.

The above results suggest that immunity had been developed in chicks treated with aureomycin, chloramphenicol, spiramycin or terramycin as a result of the primary infection.

DISCUSSION

The results obtained with aureomycin confirm other published work and those with spiramycin substantiate the findings of Cuckler and Malanga (1957). Chloramphenicol, which Majilton (1953) used with success in dog coccidiosis, erythromycin and terramycin were also active. There was no indication that aureomycin, chloramphenicol, spiramycin and terramycin interfered with the development of immunity to *E. tenella* by severely infected chicks. Their effect in milder infections was not examined.

Smith (1954) found that chloramphenicol was absorbed better than aureomycin, dihydrostreptomycin, penicillin and terramycin in chickens after oral administration. Aureomycin and terramycin persisted in the serum for a longer period than did chloramphenicol. Spiramycin is absorbed rapidly from the alimentary tract of young chicks (Inglis, 1956). The difference in absorption or excretion of these antibiotics may partly account for their differences in anti-coccidial efficiencies, and recently Barrows (1957) found that terramycin administered parenterally gave better results in coccidiosis of cattle than when given orally.

Ingram and Edgar (1951) concluded that the growth stimulating effect of aureomycin, penicillin and streptomycin was not due to a suppression of caecal coccidiosis. However, it may be profitable to examine further the effects of lower concentrations of certain antibiotics such as aureomycin and terramycin, which are regularly added to poultry feed, when fed alone, or in combination with low concentrations of established coccidiostats, against mild coccidial infections and upon oocyst production, since an anti-coccidial action may play some part in the effects obtained.

CONCLUSIONS

Aureomycin, chloramphenicol, erythromycin, spiramycin and terramycin were active against severe experimental infections of *E. tenella*. When administered before infection, spiramycin proved the most effective, and erythromycin and chloramphenicol the least active. When treatment was delayed up to 2 days after infection spiramycin was still effective.

Experiments indicated that aureomycin, chloramphenicol, spiramycin and terramycin did not interfere with the development of immunity to caecal coccidiosis in chicks infected with *E. tenella*.

ACKNOWLEDGMENTS

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STUDIES ON *ESCHERICHIA COLI* OF ANIMAL ORIGIN

3. *E. coli* Serotypes from Pigs

By

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INTRODUCTION

Studies of the aetiology of colibacillosis of calves have revealed that several serotypes of *E. coli* are frequently associated with this disease (Ingram, Lovell, Wood, Aschaffenburg, Bartlett, Kon, Roy and Sears, 1953; Wood, 1955). Some of these serotypes have also been recovered from cases of bovine mastitis and from cases of enteritis in lambs (Rees, 1958a, 1958b). A study has therefore been made of the serological identity of strains of *E. coli* from diseased pigs since it has been recorded that this organism is associated with certain enteric disorders in these animals (Gregory, 1955; Schofield and Davis, 1955; Timoney, 1956).

METHODS

A total of 259 strains of *E. coli*, all of which were from diseased pigs were kindly forwarded to me for this study. These came from different parts of the British Isles and were examined serologically. Their somatic and surface antigens were identified according to methods described by Kauffmann (1954). All the diagnostic antisera employed in previous investigations (Rees 1958a and b) were used and this number was later increased by the establishment of new serotypes.

RESULTS

The results are summarised in Table 1 in which it can be seen that very few serotypes normally associated with enteritis of calves or of human beings were found. Many strains were strongly haemolytic and a large number of them did not react with any of the typing sera then in use. Antisera were therefore prepared against some of them. It was then possible to demonstrate the frequent occurrence of three new serotypes. They were serologically distinct from one another in respect of both somatic and surface antigens. Two have since been identified by Dr. Ida Orskov of Copenhagen as 0138,K81(B) and 0139,K82(B) (personal communication). The third remains to be characterised and is still known under its catalogue number RVC2907.

Smooth grey colonies were formed by 0139,K82(B) whilst 0138,K81(B) and RVC2907 produced mucoid opaque colonies. Occasional non-haemolytic varieties were found, and this was most frequent with those from RVC2907. All three produced indole, gave a positive methyl red reaction, did not produce acetylmethylcarbinol and did not utilise sodium citrate as a sole source of carbon.

TABLE 1

THE DISTRIBUTION OF CERTAIN SEROTYPES OF *E. COLI* IN DISEASED PIGS

Disease in pigs	*Serotypes*								*Serologically unidentified*		*Totals*
	Pig			*Calf*				*Human*			
	0138K81(B)	*0139,K82(B)*	*RVC2907*	*0101,K?(A)*	*0103,K?(B)*	*0114,K?(B)*	*0117,K?*	*0119,B14*	*Haemolytic*	*Non-haemolytic*	
Oedema disease ..	9	17	18	1	2	0	1	0	11	13	72
Gastro-enteritis, with or without septicaemia	18	4	44[1]	0	0	0	0	1	31	32	130
Miscellaneous conditions	7[2]	2	6[3]	0	1	1	0	0	11	20	48
Sow milk	0	1[4]	0	0	0	0	0	0	0	5	6
Sow vagina	0	0	0	0	0	0	0	0	1	2	3
											259

[1] Seven non-haemolytic strains. [2] One non-haemolytic strain. [3] One non-haemolytic strain. [4] A non-haemolytic strain.

The sugar reactions showed minor variations. Glucose, lactose, maltose, mannitol, arabinose, sorbitol, rhamnose, trehalose and xylose were fermented with gas production by all three types within 24 hours of incubation. Inositol was not fermented by any type after 15 days. Sucrose and dulcitol were fermented promptly by 0139,K81(B) and by 0139,K82(B) and after several days incubation by RVC2907. Salicin was fermented by RVC2907 alone and then after several days. Sorbose was fermented by 0138,K81(B) promptly and by 0139,K82(B) after several days but not by RVC2907.

Unlike the strains described by Gregory (1955), these serotypes have retained their haemolytic properties unaltered for more than two years and there is some evidence that they produced a weak soluble haemolysin (Rees, 1959).

Of 72 strains from pigs with oedema disease, 44 (61·1 per cent) could be classified as one or other of the three serotypes described above. Of 130 strains from cases of gastro-enteritis, 66 (50·8 per cent) also belonged to one or other of these types. Fifteen of 48 strains derived from a miscellaneous group of conditions were similarly typed.

Seven strains were identified as serotypes known to have been associated with disease in young calves or newborn babies and their identity was confirmed by agglutinin cross-absorption tests. Variations in the fermentation of carbohydrate media from the patterns normally exhibited by the type strains occurred. One of the strains of 0·103K?(B) from oedema disease failed to ferment dulcitol while the other failed to ferment rhamnose; the one from the miscellaneous group also failed to ferment dulcitol but fermented sorbose. Strain 0117,K? failed to ferment sucrose and 0114,K?(B) fermented neither sucrose nor dulcitol. Strain 0101,K?(A) fermented salicin; 0119,B14 did not.

DISCUSSION

The outstanding result in this study concerns the high incidence of three *E. coli* serotypes in pigs: of 202 strains from cases of oedema disease or gastro-enteritis, 110 (54·5 per cent) belonged to one or other of these serotypes.

The figures in the 'miscellaneous' group suggest that the haemolytic serotypes occur less frequently in less well-defined conditions. The general picture, however, contrasts strongly with the findings of an investigation into colibacillosis of calves wherein about 30 per cent of the strains were serologically identifiable and these were distributed among eleven different serotypes (Rees, 1958a).

The aetiological significance of haemolytic strains of *E. coli* in oedema disease in pigs has been discussed at length by Timoney (1957) and Sojka, Erskine and Lloyd (1957). Strains found by Timoney were identified as 0138,K81(B) and 0139,K82(B) by

Ewing, Tatum and Davis (1958). A haemolytic strain of *E. coli* recovered from a case of oedema disease in Canada was kindly sent me by Dr. Schofield of the Ontario Veterinary College; this has been identified as RVC2907. A comparison of all three serotypes with those recovered by Sojka *et al.* (1957) shows that RVC2907 is identical with their strain OK68,K82(B), 0133,K81(B) with their OKE57 and 0139,K82(B) with their E4 (personal communication).

While it is interesting to record the incidence in diseased pigs of a small number of serotypes of *E. coli* normally associated with gastro-enteritis of calves or newborn babies, so little is known about their distribution that any discussion on their epidemiological and aetiological significance is at the moment unwise. The recovery of 'calf' serotypes from pigs may, however, be of some relevance to the epidemiology of colibacillosis. 'Calf' serotypes have already been recovered from lambs (Rees, 1958b) and it is now becoming apparent that these types may be more widely distributed than was previously believed.

CONCLUSIONS

Two hundred and fifty-nine strains of *E. coli* from diseased pigs have been examined serologically.

Three serotypes, 0138,K81(B), 1039,K82(B) and RVC2907 which are normally haemolytic, occurred in a high percentage of cases of gastro-enteritis and oedema disease. Four serotypes 0101,K?(A), 0103,K?(B), 0114,K?(B) and 0117,K?, which are usually associated with colibacillosis in calves, occurred in a small number of diseased pigs. A serotype 0119,B14, which has been recovered from cases of infantile gastro-enteritis, occurred in one of the pigs.

These records are discussed in the light of the distribution of *E. coli* serotypes in nature.

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ADDENDUM

Since the paper was submitted, the serotype RVC2907 has been established as the type strain for a new somatic and a new surface antigen 0141,K85(B). (Orskov, Orskov, Rees and Sahab (1959)).

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MUSCULAR DYSTROPHY IN EXPERIMENTAL LAMBS

By

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INTRODUCTION

Muscular dystrophy due to vitamin E deficiency or antagonism has previously been produced experimentally in lambs either by maintaining them after birth on synthetic diets which were low in vitamin E and contained unsaturated fats which antagonise the vitamin (Culik, Bacigalupo, Thorp, Luecke and Nelson, 1951; Bacigalupo, Culik, Luecke, Thorp and Johnston, 1952), or by feeding their dams during pregnancy and lactation on natural diets containing leguminous hay. In the latter case Willman and his co-workers used lucerne hay and kidney beans in a long series of successful experiments (Willman, Asdell and Olafson, 1934; Willman, Loosli, Asdell, Morrison and Olfason, 1945, 1946; Whiting, Willman and Loosli, 1949), while more recently Safford, Swingle and McRoberts (1956) reproduced the disease by feeding lucerne or clover hay from farms on which the disease was prevalent, either alone or with oats or barley.

The following is an account of the accidental production of muscular dystrophy, possibly due to vitamin E deficiency or antagonism, in experimental lambs whose dams were housed indoors and fed a diet of mixed grass hay, containing only a small proportion of clover, and oats. No unsaturated fats were added to the diet. The disease is unknown on the farm from which the hay and oats were obtained, and a clinical and pathological examination of lambs reared on this farm in 1956 and 1957 provided no evidence of muscular lesions. There does not appear to be any previous record in Britain of the production of this disease in lambs following the use of experimental diets. The purpose of our experiment was to study the effects on the health of ewes and their lambs of feeding over several years a diet with a low copper content (2 to 3 p.p.m. DW). Muscular dystrophy, predominantly affecting the myocardium, was recognised in the first lamb crop, born in 1956, only after the lambs had been slaughtered for pathological and biochemical examination. No clinical symptoms suggesting the presence of this disease had been noticed. No signs of the disease either clinical or post mortem were seen in the lambs born the following year, after they and their mothers had been given a vitamin E supplement.

MATERIALS AND METHODS

Experimental animals and diet. Twelve North Country Cheviot gimmers were bought in the open market in September 1955 and were immediately put into individual pens with concrete floors from which all accessible copper and brass fittings had been removed. For the first 7 weeks they were each fed 1½ lb. of mixed grass hay and ½ lb. of flaked maize daily. This was replaced by the low copper diet (usually 1½ lb.

hay and 1 lb. oats) in October, 3 to 4 weeks before they were mated with a Border Leicester ram. The hay was made from a mixture of cocksfoot, ryegrass, timothy and clover grown on a farm where the soil was originally deficient in cobalt and copper, and which had produced a copper deficiency disease in oats (Mitchell, Reith and Johnston, 1956). The cobalt deficiency had subsequently been corrected by the use of cobalt-rich fertiliser. Parts of the farm had been treated with copper, but it had not been applied to the fields in which the hay and oats were grown. From early January 1956 all the ewes under experiment were given 1 mg. cobalt by mouth each week in the form of an aqueous solution of Analar $CoCl_2 \cdot 6H_2O$ and half of them, forming the control group, received 100 mg. copper each week by mouth as an aqueous solution of Analar $CuSO_4 \cdot 5H_2O$. (These supplements were not given to the lambs). No further reference will be made to copper deficient and control groups, since muscular dystrophy occurred indiscriminately in both. Table 1 shows the distribution of lesions in these groups.

In April 1956 these ewes gave birth to 16 lambs, and while nursing their lambs their ration of oats was increased to 1½ lb. daily. One lamb was injured and died soon after birth. Twelve apparently healthy lambs were slaughtered for biochemical and pathological examination at the age of about 8 weeks. When muscular dystrophy was found in these lambs it was decided to feed the three remaining lambs with the above diet (1 lb. of hay and 1 lb. oats daily) without supplement to see if the condition would progress. One of these three lambs died suddenly at the age of 6 months, having been noticed to be unwilling to move on the previous day; the other two showed no clinical abnormalities and were killed for examination when 10½ months old.

As a result of these findings, and in the hope of preventing a recurrence of muscular dystrophy in the next lamb crop, a vitamin E supplement was fed to the ewes from the end of December 1956, one month after the commencement of their second pregnancy. For a month about 0·7 g. of Rovimix E (Roche Products Ltd.), containing about 70 mg. of dl-α-tocopheryl acetate adsorbed on a silica base, was given daily to each ewe by mixing it with the oats. The supplement was then increased to about 1·2 g. daily and has since been maintained at this level. Since the experiment was primarily set up to study copper deficiency and involved only a small number of animals, all were given the vitamin E supplement.

Nine of the 12 lambs which were born in April or May 1957 were in good health until they were slaughtered for examination at the age of 6 to 7 weeks. The other 3 died from haemorrhagic gastroenteritis, 2 shortly after birth and 1 at 3½ weeks old. Every lamb was given, by mouth twice weekly, about 0·7 g. of Rovimix E suspended in water.

Pathological investigations. All the lambs referred to above were examined in detail post mortem, material being taken for histological examination. A further 18 lambs varying in age from a few days to 5 months and reared under natural conditions on the farm from which the diet of hay and oats was obtained, were similarly examined during 1956 and 1957.

All tissues were fixed in 10 per cent formol saline. Cardiac and skeletal muscles were stained by Mallory's phosphotungstic acid haematoxylin method.

Haematological and biochemical investigations. Blood samples were taken from the ewes at various times for red cell and differential cell counts,

the estimation of haemoglobin (Cohen and Smith, 1919), total and inorganic phosphate (Fiske and Subbarow, 1925) and copper (Butler, 1952; Butler and Newman, 1956) in whole blood, and for the estimation of calcium (Powell, 1953) and magnesium (Denis, 1922; Hawk, 1947) in serum. The same analyses were carried out on blood samples taken from the lambs immediately before slaughter. Copper was estimated in the livers of these animals by the method referred to above.

An investigation of the plasma tocopherol levels in the ewes was begun in November 1956 before the vitamin E supplement was given, and estimations were also carried out on the two 1956 lambs which were kept until they were 10½ months old. Several difficulties were experienced in attempting to determine the low levels of tocopherol present ($\leqslant$100 μg./100 ml.) by methods which had been used by previous workers. Although the procedure used by Blaxter, Brown and MacDonald (1953) in which the final determination is made by the method of Emmerie and Engel (1938) gave good recovery values for added α-tocopherol, inconsistent results were obtained when the sample of plasma taken for analysis was increased to the quantity necessary to give a suitable amount of tocopherol for estimation. The method recently recommended by Nair and Magar (1956) was also tried, but it was not possible to obtain a reproducible calibration curve and recovery of tocopherol was extremely erratic. Eventually a modification of the micro method described by Quaife, Scrimshaw and Lowry (1949), which is also based on the Emmerie-Engel reaction, was adopted. The scale of the original method was increased so that the Unicam SP 350 diffraction grating spectrophotometer with a 4 ml. optical cell could be used, but owing to the consequent loss of sensitivity only semi-quantitative results were obtained. When tests were carried out with the Hilger Spekker absorptiometer using 0·5 ml. micro cells it was found that the radiation from the tungsten filament lamp catalysed the reduction of ferric iron in the solution containing the Emmerie-Engel reagents during the time the measurements were being carried out, so that erroneously high results were produced.

RESULTS

Macroscopic Findings

The 15 lambs examined in 1956 will be described first. Twelve were in moderately good bodily condition; 3 were rather poor. It will be seen from Table 1 that there were macroscopic abnormalities in 8. These were found in the heart in all 8 cases, in the abomasum in 3, and in the skeletal muscle in 2. The remaining 7 lambs were macroscopically normal post mortem.

Heart. This was invariably flabby. In all 8 lambs lesions were present in the right ventricle. In 2 of these lambs lesions were also present in the left ventricle, and in the left auricle in a third lamb. The lesions varied in extent and appeared beneath the smooth endocardium as white streaks and irregular patches, occasionally mottled. The streaks were arranged longitudinally from base to apex of the heart (Fig. 1). In the mildest case the lesions were confined to the papillary muscles, the moderator bands and near the origin of the valve cusps as described by Cotchin (1948), while in the other lambs most of the subendocardium of the right ven-

TABLE 1

DISTRIBUTION OF LESIONS AND THE BLOOD PICTURE IN THE LAMBS BORN IN 1956 AND 1957

1956

Lamb no.		*Age*	*Heart*	*Skeletal muscle*	*Abomasum*	*Kidney*	*Hb g./100 ml.*	*RBC x 10^6 per cu. mm.*	*Poikilocytosis*
906	copper deficient	8 wks.	—	— (macro)	—	+ (micro)	13·5	13·3	—
907		8 wks.	—	— (macro)	—	+ (micro)	12·8	11·8	—
910		8 wks.	+	— (macro)	—	+ (micro)	6·6	9·0	+ (micro)
912		8 wks.	+	—	+	—	9·8	10·5	+ (micro)
915		10½ mths.	+	—	—	—	10·6	9·4	—
916		8 wks.	+	+ (micro)	+	+ (micro)	9·8	11·6	+ (micro)
920*		6 mths.	+	+	—	+ (micro)	11·2	9·4	+ (micro)
922*		new born	—	— (macro)	—	— (macro)	N.E.	N.E.	N.E.
900	copper supplemented	8 wks.	+ (micro)	— (macro)	—	+ (micro)	12·6	12·6	—
903		8 wks.	— (macro)	— (macro)	— (macro)	— (macro)	10·9	10·3	—
904		8 wks.	+	— (macro)	—	+ (micro)	7·6	11·2	+ (micro)
905		8 wks.	— (macro)	— (macro)	— (macro)	— (macro)	9·8	10·8	+ (micro)
908		8 wks.	— (macro)	— (macro)	— (macro)	— (macro)	12·4	14·3	+ (micro)
909		8 wks.	— (macro)	— (macro)	— (macro)	— (macro)	11·5	13·0	+ (micro)
913		8 wks.	+	+	+	+ (micro)	7·9	11·0	+ (micro)
921		10½ mths.	+	—	—	—	9·8	10·4	+ (micro)

1957									
992*	copper deficient	1 day	—	— (macro)	—	—	N.E.	N.E.	N.E.
994*		3 days	—	— (macro)	—	—	N.E.	N.E.	N.E.
985*		3½ wks.	—	— (macro)	—	—	N.E.	N.E.	N.E.
986		6 wks.	—	—	—	+ (micro)	10·8	8·9	+ (micro)
993		7 wks.	—	—	—	+ (micro)	11·7	10·9	—
995		7 wks.	—	—	—	+ (micro)	12·9	13·1	N.E.
996		4½ wks.	—	—	—	+ (micro)	14·2	10·1	—
987	copper supplemented	6 wks.	—	—	—	+ (micro)	14·6	9·5	+ (micro)
988		6 wks.	—	—	—	+ (micro)	14·6	11·3	—
989		6 wks.	—	—	—	+ (micro)	13·8	13·1	—
991		6 wks.	—	— (macro)	—	+ (micro)	13·6	10·6	—
997		7 wks.	—	—	—	+ (micro)	11·7	10·8	—

N.E indicates that no examination was carried out.
+ (micro) indicates that microscopic lesions only were found.
— (macro) indicates that there were no macroscopic lesions, and that no histological examination was carried out.

+ indicates that macroscopic lesions were present.
— indicates that no abnormality was found either macroscopically or microscopically.
* indicates death from natural causes.

tricle was affected. These lesions extended to a depth of 1·0 to 1·5 mm. Patchy areas of similar appearance were seen in the substance of the ventricular walls and in the interventricular septum, occasionally affecting their entire thickness. In the most severely affected heart a subepicardial lesion extending more than half-way through the ventricular wall was seen on the surface of the right ventricle related to the coronary artery (Fig. 2).

Abomasum. The lesions were unusual, consisting of marked thickening of the wall and of the "leaves" of the pyloric part. The mucosa was white and opaque with a "morocco-leather" surface and many cystic swellings up to 1 cm. diameter which crepitated on palpation like emphysematous bullae. A few petechial haemorrhages were present throughout the mucosal surface. In one case the mucosa showed a small haemorrhagic ulcer near the pyloric orifice. From the peritoneal surface numerous bubbles could be seen through the muscle layers. Section of the abomasal wall near the pylorus showed the thickening to be due to a layer of viscid opalescent fluid in the submucous tissues between the muscularis mucosae and the external muscle coats. The gas bubbles seen from the peritoneal surface were embedded in this fluid. In 2 lambs similar lesions also occurred in the duodenum and the jejunum.

Skeletal muscle. In one lamb the muscles in the gluteal region only were affected. They were paler than normal, and the fibres were separated by oedema. In the second lamb the muscles grouped round the scapulae and femora showed intermingled pale and red groups of fibres with chalky streaks 5 to 10 mm. long visible on longitudinal section. No abnormalities were found in the cardiac or skeletal musculature or in the abomasa of the lambs born in 1957 or in those which were reared in 1956 and 1957 on the farm from which the hay and oats were obtained.

1956 Lambs

Histopathology

In addition to the 8 lambs described above, microscopic lesions were found in 2 lambs which had shown no macroscopic abnormalities. In one of these the myocardium was affected, and in the other the skeletal muscle.

Myocardium. The essential changes were an initial degeneration and necrosis of muscle cells, followed by attempts at regeneration as shown by proliferation of sarcolemmal nuclei; later the necrotic muscle was replaced by connective tissue in the form of fine collagen fibrils. Degenerate and normal muscle were sharply demarcated, the former staining a pink-tan with phosphotungstic acid haematoxylin (PTAH).

The earliest changes observed were swelling and irregularity of muscle fibre outline followed by hyaline change in the sarcoplasm, which showed increased affinity for eosin. In most cases transverse

striation persisted until the muscle fibres were no longer recognisable, but in a few areas affected fibres lost their transverse striations although the longitudinal myofibrils remained clearly visible. Beyond this stage all structure within the fibres was lost, and they appeared in PTAH stained sections as slender brown-pink granular strands. The sarcoplasm then disappeared leaving, in the subendocardial zone of degeneration in particular, a fine network resembling connective tissue but in fact composed of collapsed sarcolemmal and endomysial tubes whose nuclei had remained intact. In this subendocardial zone a few muscle fibres lying immediately beneath the endocardium were undamaged (Fig. 3), a finding similar to that of MacDonald, Blaxter, Watts and Wood (1952).

Other necrotic areas showed an intense cellular reaction consisting of proliferation of sarcolemmal nuclei along with infiltration of a few macrophages, small mononuclear cells and fibroblasts (Fig. 4). Many of the sarcolemmal nuclei were increased in size and showed prominent multiple nucleoli. In such areas multinucleated cells were commonly seen. The final stage observed was the replacement of necrotic muscle with recently formed fibrous tissue.

The subendocardial Purkinje fibres in 5 lambs (Nos. 904, 910, 913, 916 and 920 in Table 1) showed degenerative changes. The Q bands of the myofibrils disappeared, while the Z lines increased in thickness and intensity of staining with PTAH. Affected myofibrils became shrunken and deeply staining, the Z lines projecting beyond their edges, degenerating to threads with the Z lines appearing as dots at regular intervals along their length (Figs. 7, 8 and 9). Measurement showed the length of the sarcomeres in degenerated Purkinje myofibrils to be similar to that of normal sarcomeres in a state of semi-contraction.

Abomasum. Microscopic lesions were found only in the 3 lambs described as showing macroscopic changes in this organ. Numerous large cystic spaces were present in both mucosa and submucosa. In the mucous membrane these usually occurred singly, although a few were contiguous or even apparently communicating with each other, causing compression of the surrounding glandular structures. Most of these cysts appeared empty, but a few contained a small amount of granular eosinophile material. Some were lined by a flattened epithelium, some had a fine connective tissue margin, while others had no visible limiting structure. A network of similar empty spaces was also present in the submucosa, the interstices being filled by a variable amount of loose connective tissue containing foci of small round cells, some eosinophile polymorphs and a few Langhans-type giant cells with peripheral nuclei. Similar lesions occurred in the duodenum and jejunum of one of these lambs.

Skeletal muscles. The lesions were patchy in distribution, foci of degeneration being surrounded by normal muscle fibres. Affected fibres. which were at first swollen and eosinophile, quickly lost their

affinity for PTAH and stained a pink-tan colour. Transverse striations disappeared at an earlier stage than in cardiac muscle. Fragmentation and loss of sarcoplasm was followed by sarcolemmal proliferation. Necrotic fibres were not seen to be replaced by fat or fibrous tissue. Transverse sections of affected muscles showed numbers of atrophic fibres which retained their normal staining properties. There was no calcification.

Kidneys. These were examined microscopically in 11 lambs, and glomerular lesions were found in all but 3 cases.

Affected glomeruli were most commonly seen in the outer zone of the cortex. The earliest change was a swelling and proliferation of the cells of the parietal layer of Bowman's capsule, which became cuboidal or even columnar in type with large, oval vesicular nuclei. This process commenced in the cells furthest from the vascular pole of the tuft which in a few glomeruli projected into the capsular space forming an epithelial crescent. The capsular space usually became considerably distended, and the capsule itself was surrounded by fine connective tissue. Simultaneously the glomerular tuft atrophied, and in some cases disappeared completely.

It must be emphasised that in the majority of these lambs the lesions were very slight, involving only a small number of glomeruli in a section, which in some cases merely showed fine periglomerular fibrosis. The changes observed were nevertheless consistent and characteristic. The lesions, although milder in degree, were identical to those previously described in other lambs by Butler, Nisbet and Robertson (1957), except that inclusion bodies were not present and interstitial fibrosis was not a feature.

1957 Lambs

In all 9 lambs the myocardium, skeletal muscle and abomasum appeared normal on histological examination. The kidneys were the only organs to show histological lesions, which were present in every lamb over 4 weeks old. The lesions were again predominantly glomerular and similar to those already described, only more severe and widespread.

Haematology

When the 1956 lambs were divided into two groups according to the presence or absence of myocardial lesions it was apparent that both the haemoglobin values and red cell counts for the affected group were significantly lower than those for the unaffected group ($P = <\cdot05 > \cdot01$). Three of the affected lambs had haemoglobin values below 8 g./100 ml., presenting a picture of hypochromic anaemia (Tables 1 and 2). There was no anaemia (or dystrophy) in any of the lambs born the following year after the diet had been supplemented with vitamin E. The haemoglobin values for these lambs were significantly higher than those for the lambs born in 1956 ($P = <\cdot01$). A further abnormality in the blood picture was

TABLE 2

HAEMOGLOBIN VALUES AND RED CELL COUNTS FOR 1956 AND 1957 LAMBS

Year		*No. of lambs examined*	*Haemoglobin (g./100 ml.)*		*RBC (x 10^6/cu. mm.)*	
			Range	*Mean ± S.E.*	*Range*	*Mean ± S.E.*
1956	Affected lambs* ..	9	6·63–12·6	9·55 ± 0·63	9·0–12·6	10·5 ± 0·39
„	Unaffected lambs ..	6	9·8 –13·5	11·8 ± 0·55	10·3–14·3	12·3 ± 0·63
1957	None affected ..	9	10·8 –14·6	13·1 ± 0·47	8·9–13·1	10·9 ± 0·49

*Classified according to the presence of myocardial lesions.

that in 1956 poikilocytosis was present in 10 out of the 15 lambs examined including 7 of the 8 with heart lesions. The red cells showed commas, pear shapes and many bizarre forms. In 1957 slight poikilocytosis was present in only 2 lambs.

Biochemical Results

In November 1956, just before the diet was supplemented with α-tocopherol, the total tocopherol content of the plasma of the ewes and 2 of the surviving lambs which were maintained on the diet after weaning (Nos. 915 and 921) was less than 100 μg./100 ml. Similar values have been reported for the plasma of vitamin E deficient ewes and lambs (Whiting *et al.* 1949; Culik *et al.* 1951; Bacigalupo *et al.* 1952) and for the serum of vitamin E deficient calves and their dams (Blaxter, 1953). When the diet was supplemented with tocopherol the levels in the ewes showed a gradual rise until values of 200 to 400 μg./100 ml. were reached just before lambing in 1957, and samples taken after lambing showed that these levels were being maintained. Similar values were obtained for ewes which had been housed indoors at Moredun Institute and fed on a diet of hay and sheep nuts for 2 years and whose lambs had shown no signs of muscular dystrophy on detailed examination.

The blood copper levels of the ewes receiving the low copper diet showed a gradual fall from 1955 to 1957. For instance in November 1955 before tupping they ranged from 86·4 to 97·7 μg./100 ml. about a mean value of 89·6, while a year later the values were 47·7 to 79·1 μg./100 ml. with a mean value of 65·7. This fall was reflected in the liver copper values for the two lamb crops. Those for the lambs born in 1956 in which muscular dystrophy occurred ranged from 30·0 to 54·6 p.p.m. DW about a mean value of 38·9, while the range of values for the lambs born the following year which showed no signs of dystrophy was 12·3 to 32·0 p.p.m. DW and the mean value was 21·3. The corresponding values for the sheep in the control group whose diet was supplemented with copper showed no significant change. The blood levels of the ewes were within the range 77·7 to 121 μg./100 ml. (mean 92·1) and the liver values for their lambs ranged from 39·4 to 308 p.p.m. DW (mean 133).

There were no abnormalities or significant changes in the other blood constituents which were estimated viz. inorganic and total phosphate in whole blood, and calcium and magnesium in serum.

DISCUSSION

There does not appear to be any previous record in the literature of the occurrence of muscular dystrophy in lambs on experimental diets in Britain, and the available evidence indicates that the condition we have described above may have been caused by a deficiency or antagonism of vitamin E. In the first place the myopathy described here was very similar to that which has been produced experimentally in lambs and calves and which has been shown to be

prevented by the administration of vitamin E. Secondly there were no signs of the disease in the lambs born the following year after a vitamin E supplement had been given to both the ewes and their lambs. Furthermore, low levels of tocopherol were present in the blood plasma of the ewes and their lambs.

The investigations carried out by Brown (1953) suggest that the vitamin E content of the basal diet used in our experiment was probably low, but it is also possible that the vitamin may have been present in an unavailable form or that some antagonistic substance was present. Although muscular dystrophy occurred in our experimental lambs whose dams received a copper supplement, the fact that the hay and oats used in our diet was grown on copper deficient soil may be of some significance in view of the prevalence of copper (and cobalt) deficiency in cattle in areas of Finland where muscular dystrophy is also present (Broberg, 1949). Blaxter and McGill (1955) have also commented on the occurrence of muscular disease where mineral deficiency disease is common. The possible involvement of copper in the synthesis of tocopherol in pasture plants would appear to merit investigation.

Only one of the affected lambs described above showed clinical abnormality. This very low incidence of clinical symptoms may have been due to their close confinement which reduced exercise to a minimum (Warner, 1953). This demonstrates that lambs kept indoors on such diets for experimental purposes may develop extensive myocardial lesions without any clinical manifestations, and the possibility that this might affect the results of the experiment should not be minimised.

Myocardial lesions were present in all the affected lambs and invariably involved the wall of the right ventricle. A predilection for this site has been recognised by the majority of those who have investigated the pathology of both natural and experimental vitamin E deficiency in the lamb, including Metzger and Hagan (1927), Willman *et al.* (1945), Cotchin (1948), Culik *et al.* (1951), Hartley (1953) and Hartley and Dodd (1957). On the other hand Bacigalupo *et al.* (1952) found that the lesion occurred indiscriminately in both ventricles in experimental lambs and Blaxter and McGill (1955) also arrived at this conclusion from their studies of calves.

Previous workers on muscular dystrophy in lambs who have mentioned the Purkinje fibres of the heart (Bacigalupo *et al.* 1952; Hartley, 1953) have commented on their normal appearance in spite of surrounding degeneration in the myocardium. The changes we have described show that the Purkinje fibres are not immune to damage in this disease. In the calf Blaxter, Watts and Wood (1952) noted only swelling of the Purkinje fibres.

With regard to the blood picture described, low haemoglobin values in lambs with muscular dystrophy have previously been recorded by Culik *et al.* (1951) and Hartley (1953). Apart from

these reports, abnormalities of the blood have not been associated with muscular dystrophy in ruminants.

The origin of the cystic lesions which were found in the mucosa and submucosa is unknown. They too did not recur after a vitamin E supplement had been fed, but gastrointestinal lesions have never been described as a feature of vitamin E deficiency. The gross appearance of these lesions bore a certain resemblance to the oedema of the stomach wall seen in pigs suffering from bowel oedema disease, but considerable differences were apparent on histological examination. Harris and Wulzen (1950) have described a similar condition (pneumatosis) affecting the ascending colon and caecum of guinea-pigs fed diets which appear likely to have been deficient in vitamin E, due to the presence of known antagonistic substances such as ferric chloride and lard. Gas bubbles of varying size up to several millimetres in diameter were present in the submucosa and muscularis. The mucosa was intact. Cotchin (1948) has mentioned the presence of gas bubbles in the intermuscular oedema fluid in natural cases of "stiff-lamb disease".

The cause of the kidney lesions described above is also obscure. They do not appear to be related to vitamin E deficiency since they continued to occur after the diet had been supplemented with this vitamin and in fact became more severe. They show considerable similarity to the glomerular lesions of chronic pyelonephritis, but there is no indication that they are infective in origin. Harris and Wulzen (1950) in their guinea-pig experiments referred to above also found kidney lesions in which the glomeruli were in the early stages of fibrosis.

Kidney lesions which appear to be similar to those found in our experimental lambs have been previously described in lambs suffering from a natural myopathy (Hartley, 1953)*, in others affected by a toxic osteoporosis (Butler *et al.*, 1957), in hypocupraemic calves (Blakemore and Venn, 1950) and in cattle dying from "Falling Disease" in Western Australia (Bennetts, Harley and Evans, 1942)*. The latter condition, like vitamin E deficiency, is characterised by myocardial damage with fibrous replacement but is thought to be a manifestation of a dietary deficiency of copper. This association of the kidney lesion with copper deficiency can be extended since Hartley found a low liver copper value in the lambs he studied and we have recently found such values in samples from the osteoporotic lambs referred to above. However the fact that this lesion occurred in lambs from the copper-supplemented ewes, which had normal liver copper values, suggests that the association may be fortuitous.

CONCLUSIONS

The occurrence of muscular dystrophy is described in experimental lambs whose dams were housed and received a diet of hay

*Sections of affected kidneys kindly provided by Hartley and by Bennetts have been studied. The glomerular lesions in them are very similar to those described above.

and oats with a low copper content. Clinical symptoms were not apparent.

The lesions were predominantly myocardial, and in some cases were associated with a hypochromic anaemia. Pneumatosis of the abomasum and small intestine was observed in a few lambs.

The condition did not recur in lambs born to the same ewes the following year after the addition of a vitamin E supplement to the diet.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Lamb 916. Heart opened to show massive distribution of subendocardial lesions in the right ventricle.

Fig. 2. Lamb 910. Section of heart showing pale-staining area of subepicardial degeneration related to a coronary artery. PTAH × 30.

Fig. 3. Lamb 910. Subendocardial degeneration showing increased cellularity. A few surviving muscle fibres can be seen near the surface. × 30*.

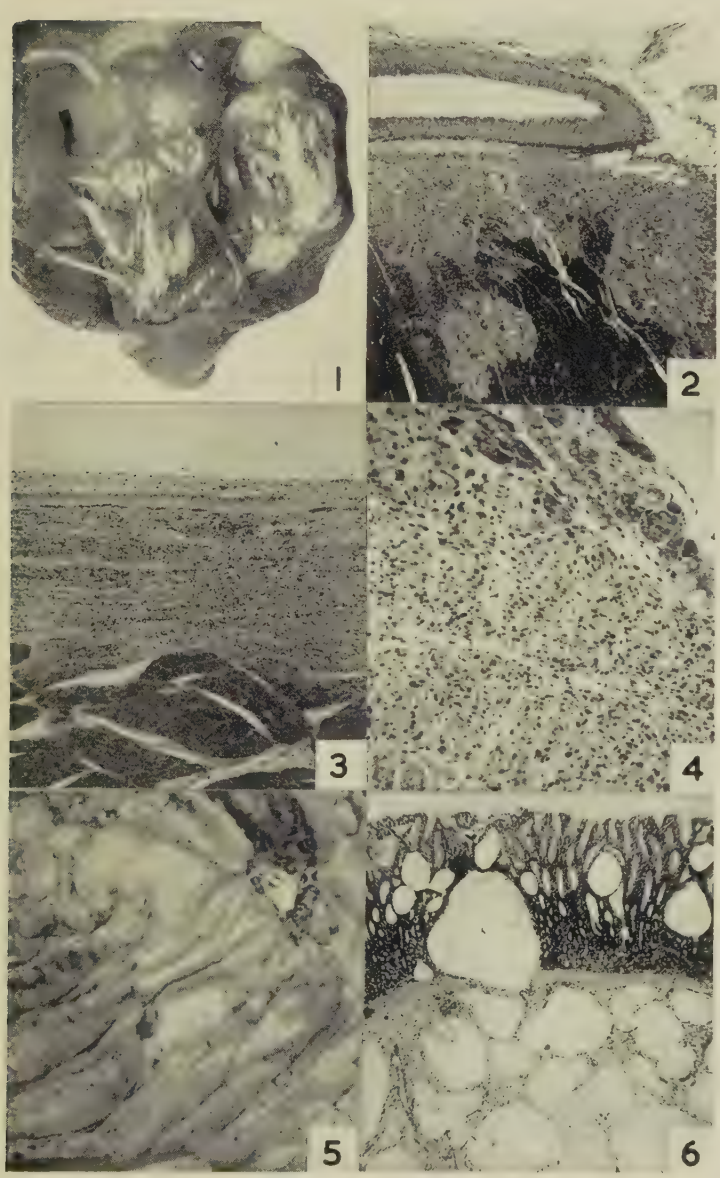
Fig. 4. Lamb 912. Subendocardial proliferation of sarcolemmal nuclei. × 120*.

Fig. 5. Lamb 912. Abomasum demonstrating thickening and opacity of mucosa. An ulcer can be seen at top right.

Fig. 6. Lamb 912. Section of abomasal wall with numerous cysts in mucosa and submucosa. H and E × 30.

Figs. 7, 8 and 9. Lamb 920. Purkinje fibres illustrating degenerative changes. PTAH × 700.

*These sections were stained by Professor A. C. Lendrum. The method has not as yet been published.



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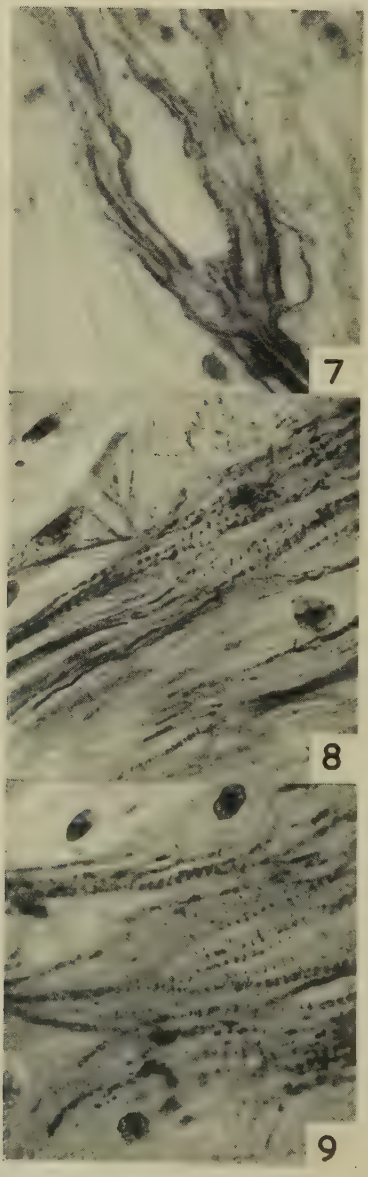
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